CONSTRUTION OF NUCLEIC ACID NANO-ARCHITECTURES BASED ON RNA TETRA-U LINKING MODULE

A THESIS

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IN PARTIAL FULFILLMENT OF THE REQUIRMENTS

FOR THE DEGREE

MASTER OF SCIENCE

BY

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Sincerely,

My N. Bui

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Abstract:

RNA nanotechnology is a rapidly emerging field and has recently received wide interest in the scientific community. The field is focused on design, synthesis, and assembly of artificial RNA nanoparticles with wide spectra of applications in synthetic biology and medicine.[1] This work demonstrates the robust properties of *in-silico* designed RNA tetra-uracil (tetra-U) 3D structural motifs for the construction of nanometer-scale nucleic acid geometries. The artificial tetra-U motif is unique and advantageous to the previous reports[2] [3] in that it possesses the special property of self-assembly and can be controlled to predictably build structures of defined size, shape, and stoichiometry. Specifically, we demonstrate the fabrication of economically favorable RNA triangular nano-scaffolds based on RNA, DNA, and hybrid RNA-DNA strands, the geometries of which were confirmed by atomic force microscope. Each of the triangle nanoparticles was thoroughly analyzed and their physicochemical properties were compared using well-established assays, including UV-melting, enzymatic degradation, immunostimulatory activity, and gene-silencing implementing RNAi technology. We found that the modulation of RNA and DNA strand composition makes it possible to engineer, in a *de novo* fashion, nanometer-scaled particles that are enzymatically resistant, thermodynamically stable, and potentially instrumental in the delivery of fluorescent probes and gene-silencing agents to cancer cells. We have also obtained preliminary data demonstrating that tetra-U motif can be used to construct other polygons made of RNA and DNA including squares, pentagons, and hexagons. This data will be crucial to further demonstrate the importance of such economically advantageous and fine-tunable hybrid RNA/DNA nanostructures to fulfill the needs of the rapidly developing field of RNA nanotechnology.

Chapter I: Literature review

I. Introduction:

Recently, nano-engineering has achieved many functions in developing and building new materials at the nanoscale level with *de novo* properties to help us understand insightfully into our nature and life.[4] Nucleic acids (DNA and RNA) are fundamental biological molecules of life and have many essential roles in gene heredity, regulation, and expression.[5] While DNA strands play as a template role to carry out genetic information and also can provide a simplest building blocks in form of a double helix, RNA is a much more complex biomolecule. Its distinct feature to self-assemble into various conformations, mimicking proteins, creates a library of catalytic RNA molecules. Moreover, RNA structural variability and the ability to self-assemble in a highly programmable fashion has created artificial RNA constructs into two-dimensional (2D) and three-dimensional (3D) geometric forms with tunable size, shape, and functions.[4]

Increasing numbers of new RNA structures are being solved and deposited each year in the structure databases (PDB and NDB). These structures reveal that RNA molecules can form diverse and often intricate 3D structures to carry out their roles. These roles generally involve specific binding to different proteins, nucleic acids (RNA or DNA), or small molecules, including drugs or metabolites. Thus, RNA nanotechnology is a rapidly emerging field and has recently received wide interest in the scientific community. In this research project, we are going to report the design and characterization of a variety of RNA nano-architectures designed *in silico* using an artificial tetra U-linking RNA module. We hypothesize that depending on the nucleic acid inclusion within the NP, we can regulate (i) the thermodynamic stability of the complex, (ii) its resistance to nuclease degradation, (iii) its immunostimulatory activity, and (iv) simultaneous delivery of multiple siRNAs targeting specific genes. This work reevaluates the

principles behind the fabrication of RNA nanoparticles by implementing the hybrid RNA/DNA nanoconstructs and will demonstrate significant economic advantages over the use of modified RNA bases.

II. An overview of nucleic acid:

1. Basic of RNA:

RNA (Ribonucleic acid) is one of the major macromolecules that are present in all living cells along with DNA and proteins. It is an important molecule with a single long chain of nucleotides. The nucleotides attach together by esterifying the 3' – OH of the sugar moiety of one of the nucleotide to a phosphate group that is joined to the 5' – OH of the adjacent sugar. According to the central dogma of biology, the principal role of RNA is to pass a message encoded in DNA to initiate the synthesis of proteins.[6] There are three well-known types of RNA: messenger RNA (mRNA) carries information from DNA to protein; transfer RNA (tRNA) transfers amino acids to ribosome, then carries them to the growing end of a polypeptide chain, each type of amino acid has its own type of tRNA; and ribosomal RNA (rRNA) associates with a set of proteins to form ribosomes. A ribosome composes a large and small subunits and will move along an mRNA to catalyze the assembly of amino acids into protein chains.[7]

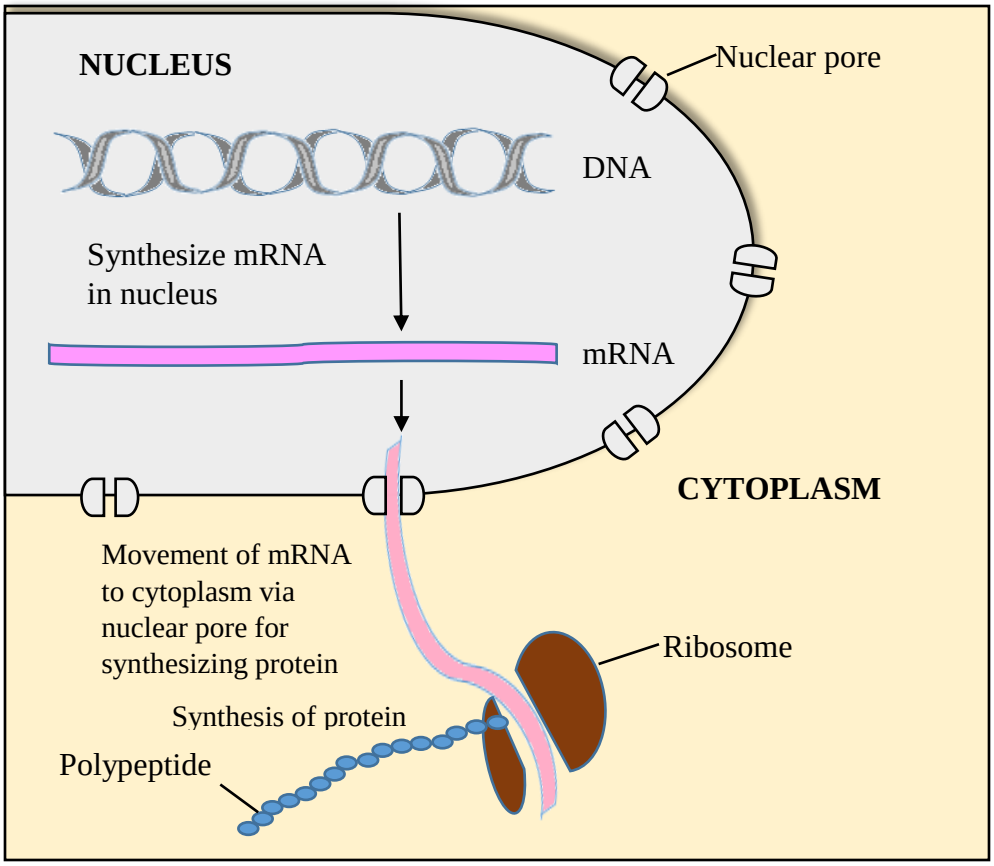


Figure 1.1: Genetic information in nucleotide sequences of DNA molecules.[7]

2. Structural features of RNA and DNA:

Unlike a double-stranded DNA, RNA is a single-stranded molecule with a shorter chain of nucleotides and plays many roles in biological functions. An important structural feature of RNA that makes a distinction from DNA is the presence of a hydroxyl group (-OH) at the 2' position of the ribose sugar (Figure 1.2 highlighted in the red box). The presence of this functional group causes RNA to adopt the A-form geometry rather than the B-form in DNA (see Table 1) and makes RNA less stable than DNA because of 2'-OH hydrolysis.[8] There are four major bases in RNA: adenine (A) and guanine (G) with a double-ringed structure called purine while cytosine (C) and uracil (U) with a single ringed structure known as a pyrimidine.[9] In the RNA molecule, not only intramolecular Watson-Crick base pairs (G pairs with C with three hydrogen bonds and

A pairs with U with two hydrogen bonds) permit the formation of short double-stranded helices, but also a wide variety of non-Watson-Crick interactions (e.g., G-U or A-A, often referred as "non-canonical" pairs) allow RNAs to fold into a vast range of specific three-dimensional structures.[1b]

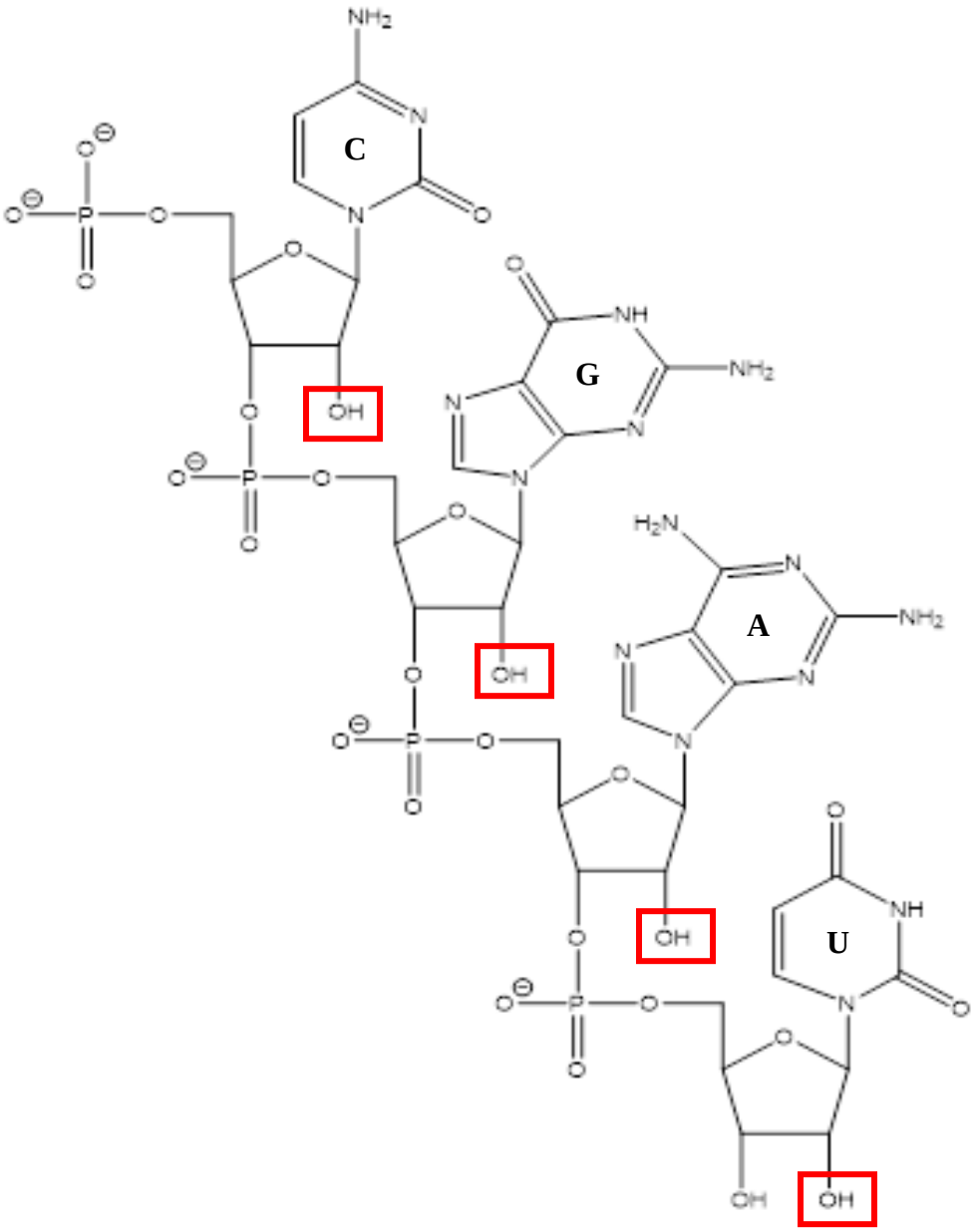
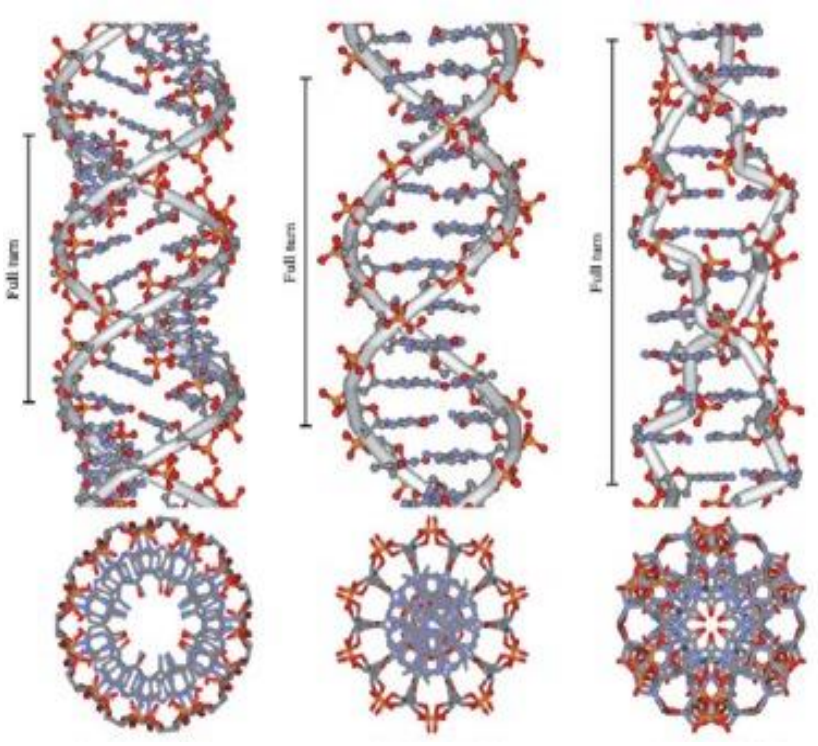


Figure 1.2: Backbone of RNA. The backbone of the nucleic acid is formed by 3' to 5' phosphodiester linkage.[8]

Table 1: Structural features of the three major forms of DNA[10] [10b] [11]

Geometry Attribute	A - DNA	B - DNA	Z – DNA
Helix sense	Right handed	Right handed	Left handed
Repeating unit	1 bp	1 bp	2 bp
Rotation/ bp	32.7º	34.3º	60º/2
bp/ turn	11	10.5	12
Inclination of bp to axis	+19º	-1.2º	-9º
Rise/ bp along axis	2.3 Å (0.23 nm)	3.32 Å (0.332 nm)	3.8 Å (0.38 nm)
Pitch/ turn of helix	28.2 Å (2.82 nm)	33.2 Å (3.32 nm)	45.6 Å (4.56 nm)
Mean propeller twist	+18º	+16º	0º
Glycosyl angle	anti	anti	C: anti G: syn
Sugar pucker	C3’ endo	C2’ endo	C: C2’ endo G: C2’ exo
Diameter	23 Å (2.3 nm)	20 Å (2.0 nm)	18 Å (1.8 nm)

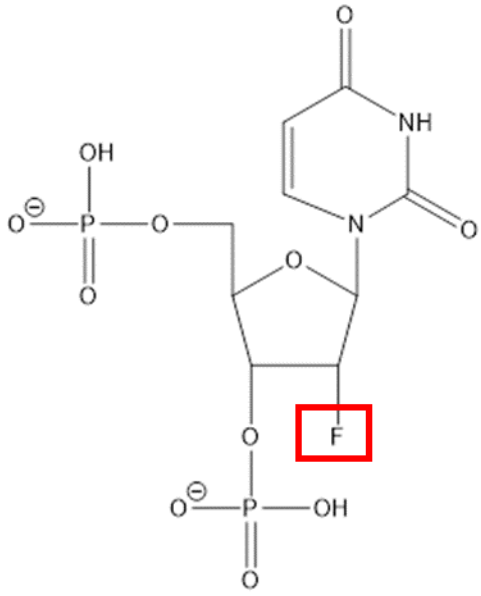


A-DNA **B-DNA** **Z-DNA**

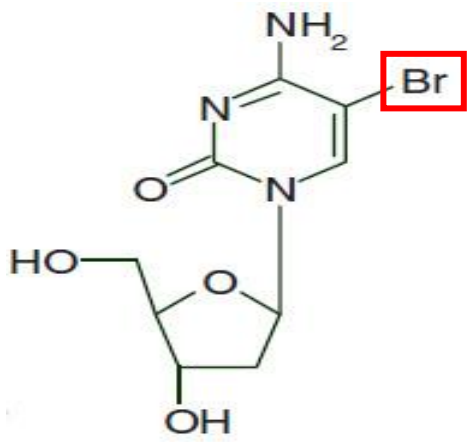
Figure 1.3: A-DNA, B-DNA, and Z-DNA conformations of DNA.[12]

3. Importance of RNA and structural manipulation:

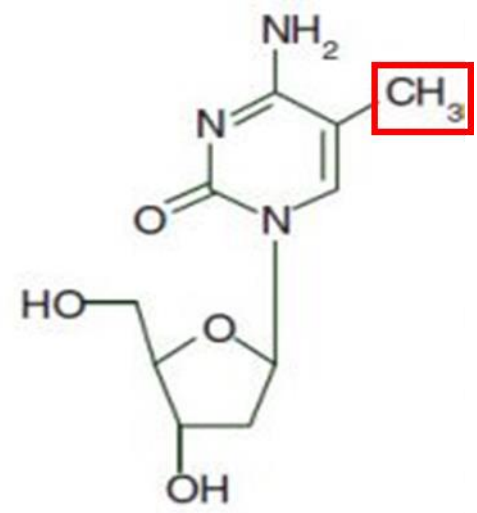
The presence of a hydroxyl group at the 2' position of the ribose sugar makes RNA less stable. Therefore, to increase the stability, there are many modified nucleotides investigated. According to Persing in 1981[13], the modified RNA results in thermal stability, reduces dynamics as well as induces an alternative folding in the secondary structure. There are some common RNA base modifications as below:



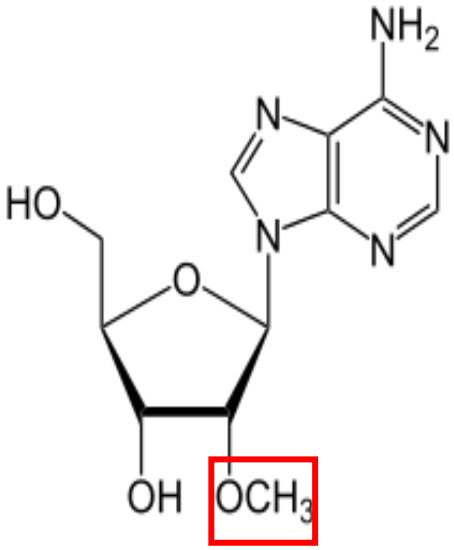
2'-Fluoro RNA oligonucleotides are used in the construction and studies of ribozymes and oligonucleotide aptamers to enhance affinity. The substitution of 2'-fluoro in an RNA monomer does not substantially change the conformation of sugar ring puckering and other structural parameters thus making this RNA oligonucleotide analogs (FRNA) as a virtual mimic of natural RNAs.[14] The internucleotide linkages in oligonucleotides with 2'-fluoro make RNA much more stable to a chemical hydrolysis at a high pH, increases binding affinity (T_M), improve nuclease resistance, stability in serum or other biological fluids.[15]



5-Bromo-2′-deoxyuridine (5-BrdU) can be considered as a thymidine analogue that can be incorporated into cellular DNA during S-phase.[16] Otherwise, 5-BrdU is used to measure DNA synthesis, label dividing cells, study cell signaling, and other processes that induce cell proliferation.[17]

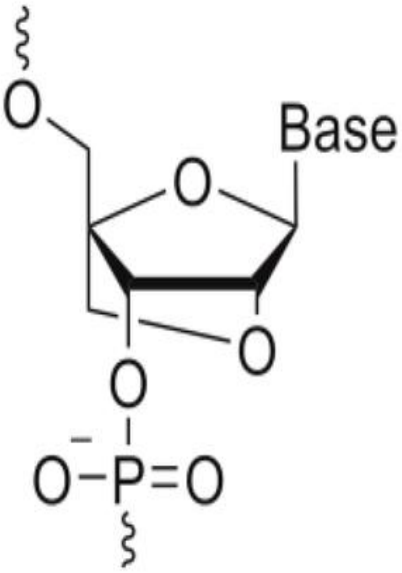


5-Me-pyridines enhance duplex stability since it improves stacking interactions. Here, a substitution of dC by 5-Me-dC, **5-Methyl-deoxycytidine (5-Methyl dC)**, will raise the melting temperature of triplex helices. In addition, the presence of 5-Methyl dC in CpG motifs also prevent or limit unwanted immune responses which is importance in antisense applications.[18] [19]



2'-O-Methyl RNA is a natural modification of RNA found in tRNA and other small RNAs that arise as a post-transcriptional modification. This type of RNA modification can increases the Tm of RNA:RNA and cause small changes in RNA:DNA stability. It is commonly used in antisense oligos as a means to increase stability and binding affinity to the target message.[20]

http://mods.rna.albany.edu/mods/modifications/view/88



Locked Nucleic Acid (LNA) is another type of modified RNA nucleotide synthesized by the group of Jesper Wengel in 1998 containing a 2'-O, 4'-C methylene bridge which favors RNA A-type helix duplex geometry.[21] This modification can increase Tm, increase affinity for its complementary strand compared to traditional DNA or RNA oligonucleotides, and is also very nuclease resistant. Therefore, it is an ideal for the detection of small or highly similar DNA or RNA targets.[22] [23]

4. Common types of RNA and their functions:

Among well-known mRNA, tRNA and rRNA, there are various different types of RNA, each playing a specific role in cell including: Small nucleolar RNAs (snoRNAs) serve as director of rRNA methylation, pseudo-uridylation, and regulatory roles.[24] Small interfering RNA (siRNA) has 20–25 nucleotides and interferes with gene expression through the cleavage of mRNA by a protein–RNA complex called RNA-induced silencing complex (RISC).[25] [26] This type of RNA is becoming the next generation in cancer therapy. MicroRNA (miRNA) is a small non-coding RNA molecule containing about 22 nucleotides and found in plants, animals, and some viruses. It functions in RNA silencing and post-transcriptional regulation of gene expression. RNA aptamers recognize specific ligands.[27] [28] Ribozymes have enzymatic activity. They have significant therapeutic potentials capable of regulating gene function by intercepting and cleaving RNA substrates, such as mRNA, or the viral genome of RNA containing a sequence with functions similar to that of antibodies in their ability to recognize specific ligands complementary to the catalytic center of the ribozyme.[29] [30] Riboswitches are a family of oligonucleotides (organic compounds, nucleotides, or peptides) through the formation of binding pockets.[31] [32] [33]

The production of RNA molecules is known as transcription which utilizes DNA-dependent RNA polymerase. The schematic diagram highlights this process in figure 1.3:

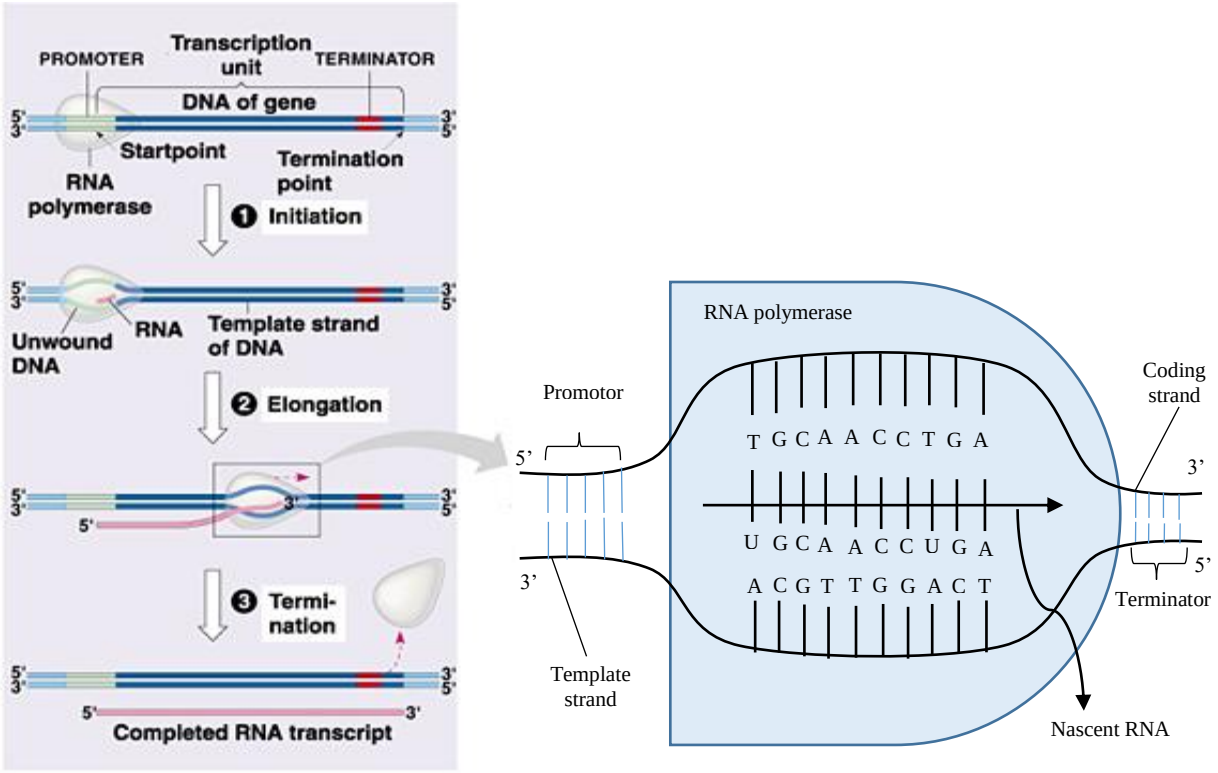


Figure 1.4: The stages of transcription: initiation, elongation, and termination.[9]

(http://course1.winona.edu/kbates/Bio241/making.htm)

There are three major steps involved in the RNA transcription process:

Initiation: During this step, RNA polymerase binds to the specific DNA sequence (promoter region) and causes the DNA strands to unwind. Then the polymerase initiates RNA synthesis at the start point on the template strand.[9]

Elongation: In this step, the polymerase moves downstream, unwinding the DNA and elongating the RNA transcript 5' → 3'. In the wake of transcription, the DNA strands will re-form a double helix.[9]

Termination: The RNA transcript is released and the polymerase detaches from the DNA.[9]

III. The emerging field of RNA Nanotechnology:

1. Definition of RNA Nanotechnology:

RNA nanotechnology is a branch of nanotechnology that utilizes RNA biopolymer as a primary material to design and fabricate *de novo* functional nanostructures with wide spectra of applications from medicine to electronics to modernize our world. The field of RNA nanotechnology has been working on applicational development and use of RNA nanoparticles. RNA is primarily composed of four nucleotides: adenine(A), cytosine (C), guanine (G) and uracil (U), giving RNA the simplistic design of DNA. This concept allows for the construction of molecules with easily predictable structures with defined shapes and stoichiometry. However, unlike DNA, ribonucleic acids allow for non-canonical base pairing as well as RNAs contain bulges, loops, and interactions between bases on separate strands creating kissing loops.[1b] The diversity in base interactions found in RNA provides a wide diversity in folding and structure similar to proteins.[34]

2. Significance of RNA for nanotechnology:

Furthermore, with the diverse folding of RNAs brings a variety of functionalities and actions. Traditionally, RNA is considered as a temporary intermediate of DNA for the translation of proteins; however, it is now well understood that RNA holds many functionalities within the body and non-coding RNAs play important roles through gene expression and regulation. Such examples of these RNAs are ribozyme[29-30], riboswitches[31-33], small interfering RNA (siRNA)[25-26], micro RNA (miRNA)[27], ribosomal RNA (rRNA)[7], small nucleolar RNAs (snoRNA)[24], small nuclear RNA (snRNA)[35], and RNA aptamers[28]. The simplistic design, yet diverse structuring and functionality of RNA has made it attractive to many scientist for applicational uses such as the treatment of cancers and viral infections. Due to the nature of RNAs, RNA nanotechnology

provides several benefits over competing nanotechnologies utilizing proteins, DNA, and polymers[17 38]: 1) as stated earlier, RNA can be produced with defined shape and stoichiometry as well as high reproducibility. 2) RNA can target specific cell groups by targeting cell surface receptors through the use of RNA aptamers without inducing antibody production, allowing for repeated delivery and therapy. 3) RNA nanoparticles that have been produced have a size range of 10-50 nm, the perfect size to be retained within the body and pass through leaky blood vessels in cancer tumors by Enhanced Permeability and Retention (EPR), as well as cell membranes by cell surface receptor endocytosis. 4) RNA can be created to harbor multiple therapeutic elements by utilizing branch scaffolds and bottom-up construction. 5) RNA is known to have a higher thermostability over DNA with a higher melting temperature and more negative Gibbs free energy of formation. 6) RNA is treated as a chemical drug rather than biological entity, facilitating FDA approval.

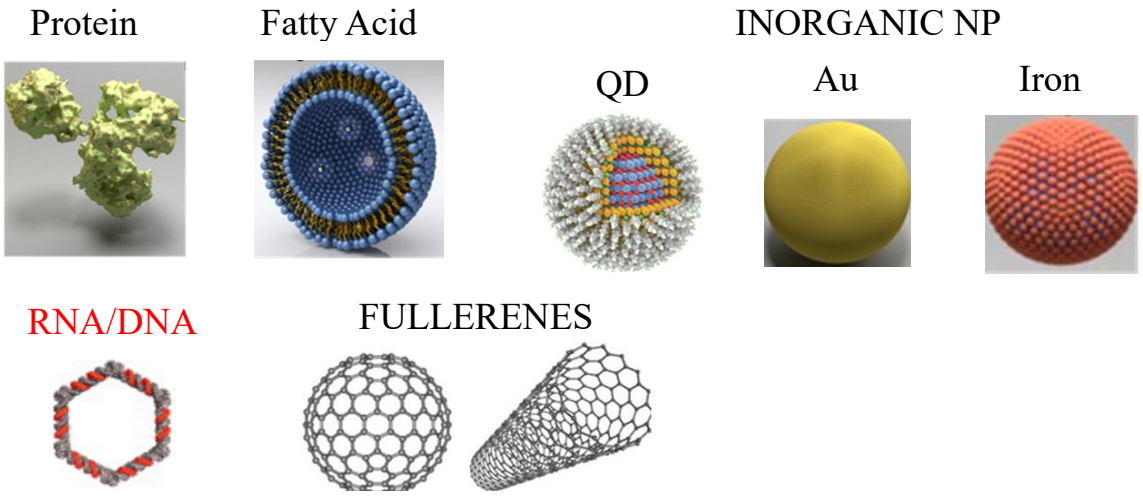


Figure 1.5: Examples of nanoparticles

There are several nanoparticles in life as shown in figure 1.4 but nucleic acid (RNA or DNA) nanoparticles should be more advanced in the cell than other. First, as mentioned in the previous, RNA nanoparticles have small size in between 10-50 nm, this is the perfect size to be

retained within the body and pass through leaky blood vessels in cancer tumors as well as the cell membranes. Second, nucleic acid nanoparticles are not poisonous, they will not be eliminated from the body by liver and therefore, they can be used as the platform for therapy and diagnostic probes in nanomedicine. Finally, nucleic acid carries negatively charge as same as cell membranes and they need ligands to take up into the cell.

3. RNA Self – assembly strategies, bottom-up and architectonic approach

RNA exhibits rich chemical, structural, and functional diversities for instance mRNAs carry information that directs protein syntheses, rRNAs fold and assembles into ribosomes, ribozymes catalyze chemical reactions, aptamers can specifically bind to ligands, microRNAs and siRNAs regulate gene expression. The ability of RNA to fold on itself can be used to design and manipulate to produce a variety of different nanostructures. RNA self-assembly generally refers to the spontaneous process by which a pre-existing sequence of nucleotides forms an organized structure consisting of a specific network of local noncovalent interactions (i.e., hydrogen bonding and stacking between nucleotide) and taking advantage of both canonical WC and noncanonical base pairs.[35] [1a] Nanotechnology uses either top-down approaches or bottom-up assembly to create nano-scale structures.[1b] Self- assembly of RNA nanoparticles is a bottom-up approach since it requires the use of programmable, addressable and predictable building blocks to generate products with defined physical, chemical, and biophysical properties as well as the diversities in both structures and functions.[1b] [36]

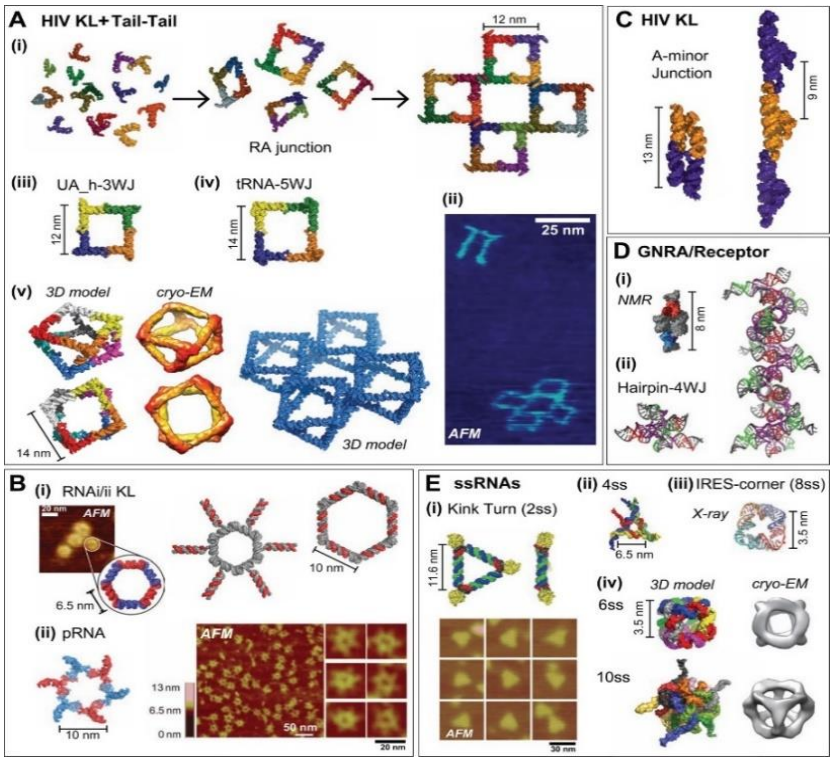


Figure 1.6: RNA nanostructures constructed using RNA self-assembly.[1a] This figure has been adopted with permission from Grabow, W. W.; Jaeger, L., RNA self-assembly and RNA nanotechnology. *Accounts of chemical research* **2014,** *47* (6), 1871-80.

(A) Several tertiary interactions directing a 90° bend between adjoining helices have been used to generate tectosquares.

(B) Hexagonal nanoparticles built from the RNAI/IIi kissing loop and the pRNA.

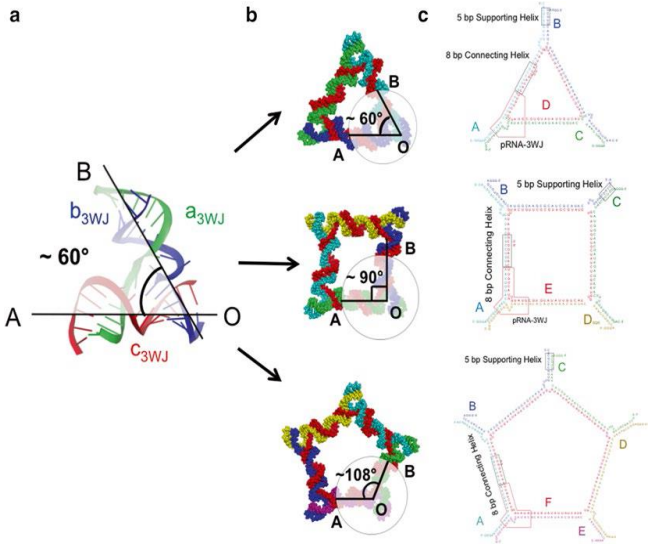
(C) RNA particles and fibers incorporating the HIV KL and A-minor Junction.

(D) Particles (i) and fibers (ii) using GNRA loop-receptor tectoRNAs.

(E) RNA nanoparticles built using the ssRNA strategy.

There are several methods to construct RNA nanoparticles from RNA properties.[1b]

i. Construction RNA polygons by Tuning Interior RNA three-way junctions (3WJ):



a) Crystal structure of pRNA-3WJ showing internal ∠AOB angle used to construct polygons.

b) 3D models of RNA polygons with internal angles highlighted with a circle.

c) Sequences and 2D structures of RNA polygons showing connecting helices and supporting helices.

Figure 1.7: Structure of Phi29 packaging RNA (pRNA) 3WJ and RNA polygons.[2] This figure was taken with permission from Khisamutdinov, E. F.; Bui, M. N. H.; Jasinski, D.; Zhao, Z.; Cui, Z.; Guo, P., Simple Method for Constructing RNA Triangle, Square, Pentagon by Tuning Interior RNA 3WJ Angle from 60° to 90° or 108°. In Methods in molecular biology, Springer Science+Business Media: New York, 2015.

There are two significant disadvantages by using natural RNA motifs to create RNA polygons: RNA tectonics using the **natural** occurring RNA motif 3WJ can only create a nanoparticle from a native angle. For instance, a 60° native angle can only use to generate a triangular nanoparticle or a 90^{o} angle is only used for square shape. Hence, any manipulation of these motifs, such as stretching to larger angles from a smaller native angle, will result in the structural instability.[3, 37] Therefore, in this project we proposed a design that can give RNA polygons with more flexible angles such as a hexagon, heptagon and even octagon in the following section.

ii) RNA nanoparticle design using 4x4x4 tetra-U helical linking module:

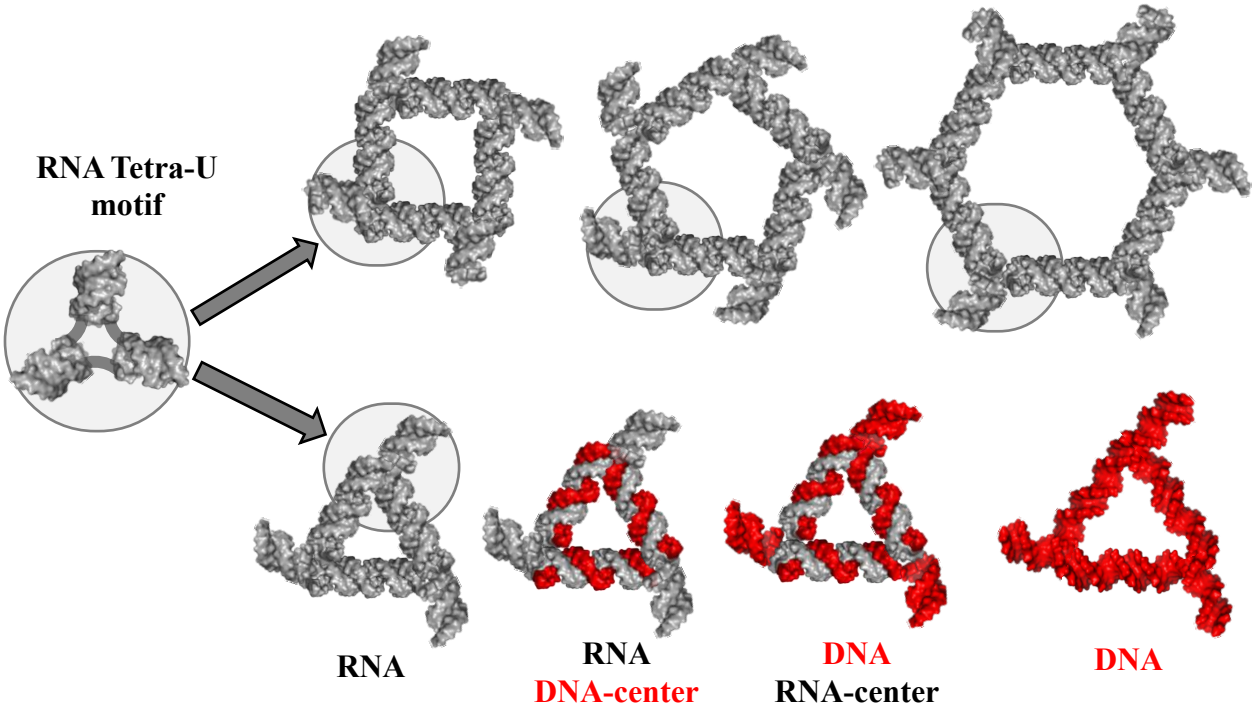


Figure 1.8: Model structures of RNA polygons using tetra 4x4x4 Uracils linking.

In this work, we proposed to use an artificial RNA module containing a bulge 4x4x4 Uracils flexible region with the corresponding number of helical arms to design RNA nanoparticles. Figure 1.7 demonstrates the tetra-U RNA module which will be implemented to construct nanoarchitectures possessing an equilateral triangle shape as well as permit the construction of other polygons including a square, a pentagon and a hexagon without any loss of structural stability of the nanostructures compare to the nature RNA motif . Why is Uracil and where are they in the RNA strands? The 4x4x4 tetra-U liking will be placed in the corners of the individual polygons and flanking C-G base pair. Uracils (U) were selected rather than other nucleotides for use in the single strand corner linkers due to their small size and they can increase the resistance of the full nanoparticles to ribonuclease.[1b, 38] This design can allow fabricating the nanoparticles from both RNA and DNA strands, as well as their mixtures resulting in a hybrid DNA/RNA. In

this project, we only focused on the construction and characterization of triangular nanoparticles including RNA, DNA, and two hybrids RNA/DNA.

IV. Construction of RNA nanoparticles:

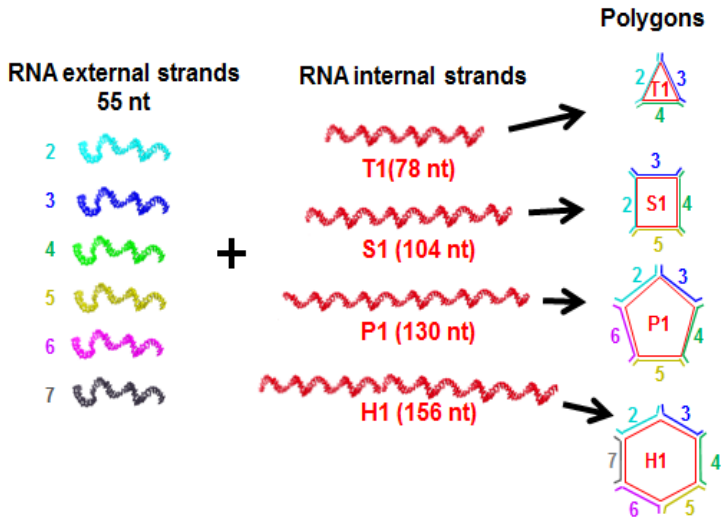


Figure 1.9: Construction of RNA polygons.[2]

Each polygon is composed of a different number of RNA strands categorized as short strands (external) and long strands (internal). When increasing the number of external strands and the propagation of the internal strand, the tension on the inter-helical will increase to 60º, 90º, 108º and 120° allowing for the 2D formation of a corresponding triangle, square, pentagon and hexagon shapes.[2]

V. Applications of RNA nanotechnology

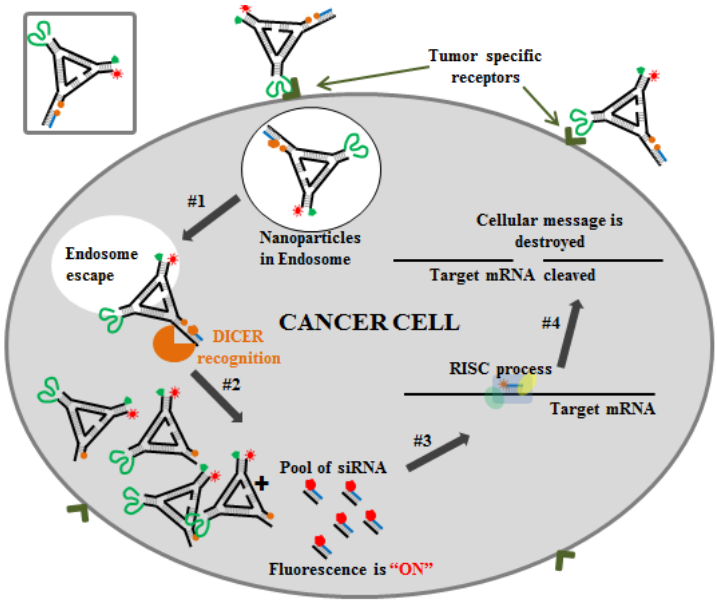


Figure 1.10: Application of RNA nanotechnology

The main aim of RNA nanotechnology is to understand the structure-to-function relationship of each RNA polygon. Therefore, develop RNA-based nano can deliver the platform for therapy and diagnostics probes. It has potential in the treatment of cancer, viral infection, genetic diseases and other applications in nanomedicine such as cell recognition and binding for diagnosis, targeted delivery via receptor-mediated endocytosis, intracellular control, and computation via gene silencing and regulation, nuclear membrane penetration, and brain blood barrier passing.[25 29]

Chapter 2: Materials and Methods

I. Computer aided design of RNA nanoparticles:

1. 3D – Swiss Pdb Viewer:

This is a powerful molecular graphic program that helps us learn how to obtain, manipulate, and analyze RNA structures, in which, we can build models simply from scratch by giving a nucleic acid sequence. The program will help to create RNA structures in 3-dimensions, find hydrogen bonds within RNA or between RNA and ligands, obtain angles and distances between atoms, allow us to examine electron-density maps from crystallographic structure determination, judge the quality of maps and models so that we can view several models simultaneously.[39]

2. Sequence optimization using *Mfold*:

By using this software, we can submit a single nucleotide sequence as input, then it will help to predict the secondary structure of single-strand nucleic acids RNA and DNA; predicts a minimum free energy, ΔG, as well as minimum free energies for folding that must contain any particular base pair.[40] Secondary structure prediction of nucleic acids is straightforward because its formation in nucleic acids follows the simple rule of Watson - Crick base pairing: A pairs with T or U using two H-bonds whereas G pairs with C using three H-bonds, thus G-C pairs are more stable than A-T pairs. Among the potential secondary structures, the one with the lowest free energy is the most likely structure.[41]

II. Polymerase chain reaction (PCR):

1. DNA primer designs:

Designing primers to perform Polymerase Chain Reaction (PCR) is the first step needed to be done. The primer is a short DNA sequence that is complementary to the template and is used to

initiate DNA synthesis. There are two primers for DNA synthesis: forward primer is complementary to the beginning of the double strand DNA and the reverse primer is complementary to the end of the sequence.[42]

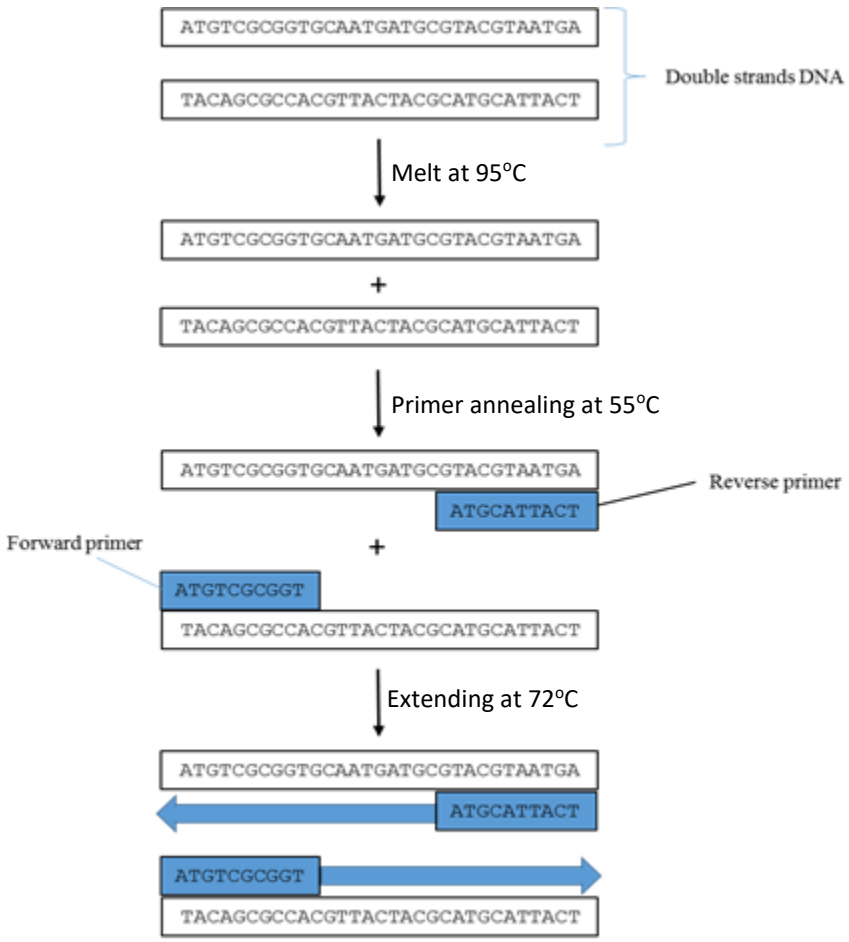


Figure 2.1: Schematic diagram of first round PCR reaction and primer design

Primers design procedure:

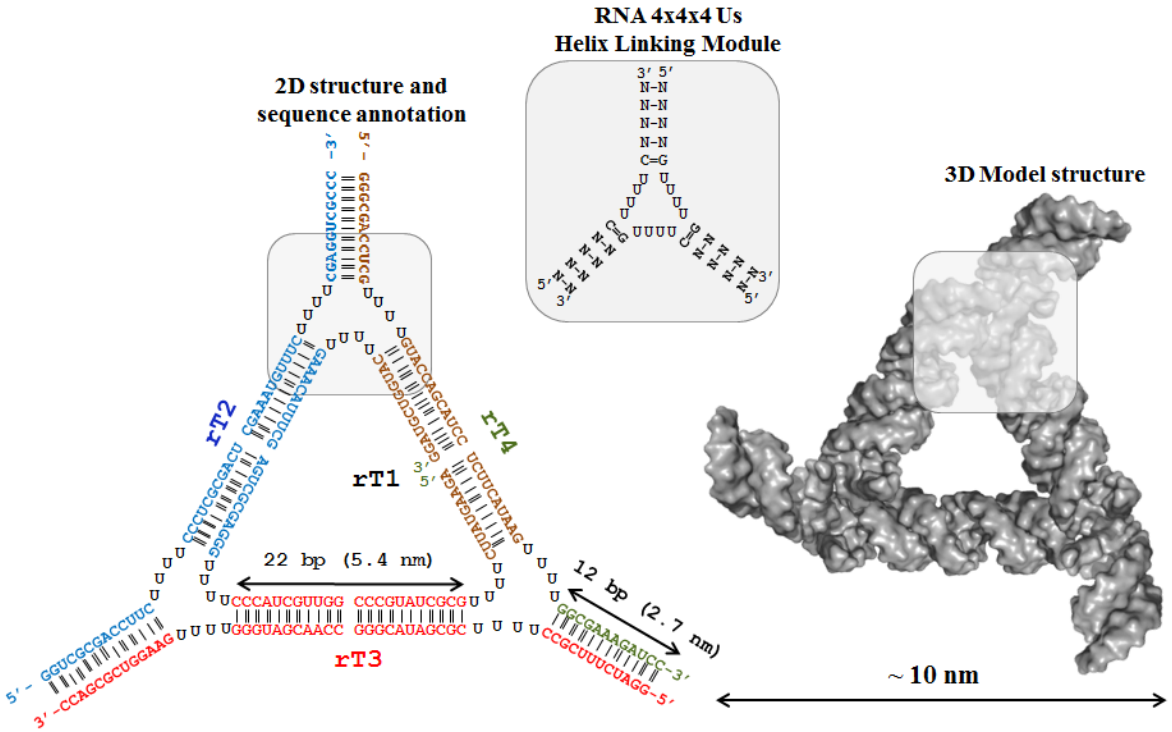


Figure 2.2: RNA triangle nanoparticle design

1. Start from 5' to 3' with 2 GG as the first nucleotides, apply appropriate numbers of RNA external strands and the propagation of internal strands with appropriate numbers of nucleotides. The edge of each nanoparticle is generated randomly by 22 base pairs nucleic acid duplexes in a length of 5.4 nm, and have predicted $\Delta G = -37.0 \pm 3.0$ kcal/mole per double helix given by *Mfold* program. Each apex of the nanoparticles contains two complete RNA turns resulting in a length of 11 bp corresponding to 2.7 nm to additionally stabilize the nanostructure and includes at least two GC/GC base pairs at the end to prevent the duplex from opening on the apex. The RNA 2D structure will be then submitted to the *Mfold* program to predict the formation of alternative stable conformations and prevent misfolding of the sequences. (See the Appendix for Nucleic Acid sequences)

2. Getting the DNA template strands: Use Sequence editor program (http://www.fr33.net/seqedit.php) to convert U nucleotide on RNA strands to T nucleotide on the DNA follow by TATA box (TAATACGACT CACTATA) to complete the DNA template.
3. Design Forward (FRW) primers: Start from the beginning except the TATA box on the DNA template, choose a sequence from 18 to 22 nucleotides and that will be the FRW primers (shown in red).
4. Design Reverse (REV) primers: This primer is complementary and inverse to the FRW primer and placed to the end of the sequence.

2. PCR reactions:

PCR reaction is a way by which we can synthesize many copies of our DNA of interest with the help of polymerase and a programmable machine. Polymerase is an enzyme that synthesizes nucleic acid using nucleic acid template. DNA polymerases are unable to initiate DNA synthesis on their own; they need a short nucleotide called a primer which was explained in the previous section. The PCR reaction has a cycle of three steps that can change the temperature from 95^{o}C to 68^{o}C.[43]

- Denature DNA: This step will heat the DNA to 95^{o}C. The hydrogen bonds that hold the DNA strands together in a helix will be broken, and allow the double strands to separate.[43] [44]
- Primer annealing: After heating, the mixture is then cooled to 55^{o}C. This will allow the primer to anneal to their complementary sequence in the template DNA.[44]
- Extension: The reaction is now heated to 72^{o}C which is the optimal temperature for DNA polymerase to react. The DNA polymerase will extend the primers by adding nucleotides onto the primer to create new DNA strand that is complementary to the DNA template.[44]

- This process is repeated for 25 – 35 cycles. Once the reaction is completed, the final temperature is set at 4^{o}C for an hour to maintain products before removing the tubes from the machine.[44]

Procedures:

There are several commercial PCR reaction kits available to use. Here, we decided to use the kit from Promega Corporation, GoTaq®FlexiDNA Polymerase.

Equipment needed:

1. PCR machine
2. Sets of micropipettes
3. PCR tubes

Reagents needed:

1. 100 μM stock concentration DNA primers for *in vitro* RNA transcription dissolved in double-deionized water (dd H_2O).
2. Kit from Promega Corporation, GoTaq®FlexiDNA Polymerase.

To set up PCR reaction, carefully follow the recommended protocol from Promega Co, summarized below:

Reagents	Volume
5x GoTaq buffer	20 µL
25 mM $MgCl_2$	10 µL
2.5 mM dNTPs	8 µL
Forward primer	2 µL
Inner 1 (if needed)	0.5 µL
Inner 2 (if needed)	0.5 µL
Reverse primer	2 µL
TAQ enzyme	1 µL
Deionized water	57 µL (without inner 1 and 2) 56 µL (with inner 1 and 2)
V_{tot} = 100 µL	

To set the control, replace 1 µL TAQ enzyme with 1 µL dd water

On a thermal cycler set up the following steps:

Step #1: 95 °C for 5 min.

Step #2: 95 °C for 1 min.

Step #3: 55 °C for 1 min.

Step #4: 72 °C for 1 min.

Step #5: Repeat Steps #2, 3, and 4 25 times.

Step #6: 72 °C for 5 min.

Step #7: 4 °C for 12 h

3. PCR products purification:

There are two ways to purify DNA after PCR reaction:

1st way (Using spin column): Using QIAquick PCR Purification Kit (http://www.qiagen.com/, cat #28706).

Spin column contains a solid phase of silica membrane and allows the nucleic acid to bind under certain conditions.

1. Combine 100 µL of PCR solution and 500 µL of PB buffer to the spin column (1:5 ratio), centrifuge at 13000 X for 1 minute, then discard the supernatant. In this step, the nucleic acid will bind to the silica membrane when the binding solution passes through the column.
2. Add 700 µL 1 x PE EtOH washing buffer to the column, centrifuge at the same rate as above for 1 minute, discard the supernatant and the empty column is then put to the centrifuge again for another 1 more minute. By this way, any remain impurities will be removed, and only the nucleic acid sticks to the silica membrane.
3. Transfer the column to the 1.70 mL centrifuge tube, add 30 µL deionized water and centrifuge for 1 minute. Now, water is as elution buffer and will remove the nucleic acid from the membrane. The nucleic acid is collected to the centrifuge tube.
4. Determine DNA concentration using Nanodrop 100 Spectrophotometer instrument at 260 nm. Usually, DNA concentration is 0.1 – 0.2 µg/µL after this purification.
5. Check the approximate length of purified DNA templates on 2 % agarose gel using 1 x TAE buffer prior to RNA transcription reaction.

2nd way (EtOH precipitate): (This way will not remove any enzyme)

1. Add 300 µL 100% cold EtOH (at least 2.5:1 volume ratio) and 10µL of 3 M sodium acetate (1:10 volume ratio) to 100 µL PCR solution, vortex and place at -20^{o}C for 1 hour or overnight.

2. Centrifuge at 13500 X for 30 mins, discard liquid and keep the pellet. Wash with 500 µL 75% cold EtOH, centrifuge for 10 mins at the same rate, dry under vacuum, and redissolve in 20 µL dd H_2O.
3. Determine the concentration of DNA by using Nanodrop 1000 Spectrophotometer and store at -20^oC.

Nanodrop spectrometer:

1. Turn on spectrometer and choose nucleic acid assay setup.
2. Blank spectrometer with 2 µL Millipore water.
3. Measure the absorbance of a water sample to ensure the spectrometer is clean.
4. Use 2 µL DNA sample to measure the concentration.
5. Record DNA concentration.

4. DNA Quality analysis on agarose gel electrophoresis:

Equipment:

1. 100 mL corked Erlenmeyer flask
2. Gel documentation system

Solution needed:

1. Tris-acetate-EDTA (TAE) buffer, pH = 8.0; 1× buffer composition: 40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA.
2. 10% EtBr
3. Agarose gel powder
4. Agarose gel loading dye
5. Purified DNA
6. Low molecular weight DNA ladder

Set up 3% Agarose gel electrophoresis:

1. Place 3g agarose to a corked Erlenmeyer flask.
2. Add 100 mL 1 x TAE buffer using graduated cylinder.
3. Heat for 1 minute in microwave, swirl flask and briefly cool at room temperature.
4. Add 1 µL EtBr (Wear glove since this is mutagenic); swirl flask for mixing.
5. Pour liquid into gel cassette and position combs. It takes about 20 mins for the gel to set.
6. When the gel is set, load 2 µL DNA samples and molecular weight ladder (mix with 2 µL loading dye).
7. Run the gel in 0.5 x TAE buffer at 100V for about 45 mins to check the approximate length of purified DNA.
8. Take picture of the gel using a gel documentation system.

III. RNA transcription using "home-made" T7 RNA polymerase:

1. Overexpression and purification of T7-RNA Polymerase for RNA synthesis *in vitro*

1.1. Introduction:

As mentioned in chapter 1, transcription is the synthesis of RNA using a molecule of DNA as the blueprint. Typically, there are two ways to synthesize RNA: chemical synthesis and enzymatic synthesis. When a large amount of RNA is desired, (e.g., for making RNA nanoparticles to study therapeutic properties *in vivo*) it is an advantage to use chemical synthesis based on phosphoramidite technology. However, one of the major limitations of chemical synthesis is the production of long RNA polymers, as it becomes very difficult to synthesize individual RNA strand longer than 50 nucleotides.[45] Therefore, the transcription reaction using T7 RNA polymerase will be a useful method which requires DNA template to produce RNA polymer and have an important role in the synthesis of RNA *in vitro* as well as for protein

overexpression *in vivo*. One of the benefits of T7 RNA pol is that it can produce milligram quantities of natural RNAs *in vitro* transcription reactions ranging from 30 – 30,000 nucleotides and allow the preparation of mutant RNAs that cannot be obtained from living cells.[46] T7 RNA polymerase has a size of 98 kD. In this section, the overexpression and purification of T7 RNA polymerase enzyme as well as the optimized transcription condition to produce a large amount of RNA nanoparticles are described in some detailed information.

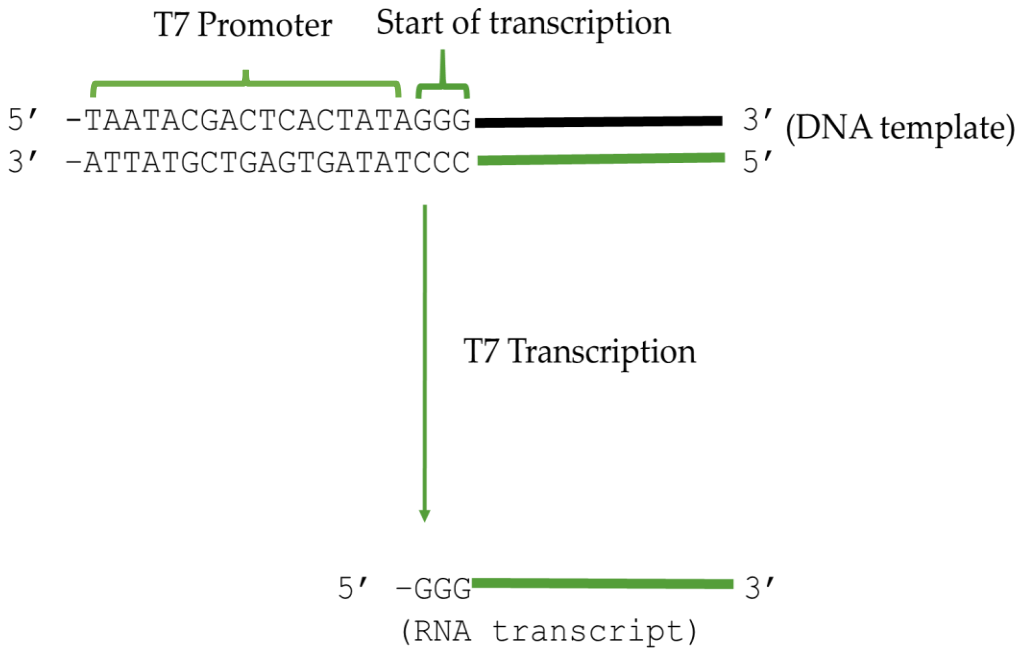


Figure 2.3: T7 RNA Polymerase recognizes its promoter and starts transcription at the final G in the promoter sequence. The polymerase then transcribes using the opposite strand as a template for 5'- 3' transcription.

1.2.Materials preparation:

a) Culturing of cells:

- **Luria agar medium:** For 1L total volume, 10g Tryptone, 5g Yeast extract, 10g NaCl and 15g of Agar were dissolved in 960 ml of dd H_2O. The solution was then sterilized in an autoclave for 45 mins using liquid cycle. After cooling down and before the medium started to be solid, add 1 ml of ampicillin stock solution to 100 µg/ml final concentration,

swirled to homogeneity. The solution was then poured into Petri dishes up to half of the dish and left until it became solid. The *E. coli* (stored at -80^{o}C) carrying a recombinant gene encoding His6-tagged T7 RNA Polymerase would be thawed on ice and spread onto Petri dishes containing medium by using inoculator and incubated 24 hours at 37^{o}C. The *E. coli* cells encode for this protein will resist ampicillin and survive on the medium containing ampicillin. The small and circular cell colonies will be observed after that.

- **Luria Broth solution:** Mix 20g tryptone, 20g NaCl, and 10g of yeast extract in dd H_2O up to 2L as the final volume. The solution was then also sterilized in an autoclave for 45 mins using liquid cycle as the Luria Agar medium.
- **Prepare 100 µM of IPTG:** Weight out 0.048 g of 0.1M stock IPTG, and dissolve in up to 2 ml of dd H_2O.

b) Buffer preparation (100 ml):

- **1X Binding buffer:** 50 mM Tris, 100 mM NaCl, 5 mM β-Mercaptoethanol, 5% glycerol, and **1 mM** imidazole.
- **Washing buffer:** 50 mM Tris, 100 mM NaCl, 5 mM β-Mercaptoethanol, 5% glycerol, and **10 mM** imidazole
- **Elution buffer:** 50 mM Tris, 100 mM NaCl, 5 mM β-Mercaptoethanol, 5% glycerol, and **100 mM** imidazole.
- **3 mL of Ni-NTA-agarose resin (Qiagen):**

Ni-NTA affinity chromatography has the efficiency to purify the recombinant his-tagged proteins from bacterial cells. Affinity chromatography is one of the purification methods which uses the specific binding interaction between molecules. A particular ligand is immobilized to a solid phase so that the molecules having specific binding affinity will bound to the ligand when

the complex mixtures pass through the column and the other contaminants are washed away resulting in its purification from the original sample.[47 48] In this case, the overexpression of His6-tagged T7 RNA polymerase is rapidly purified using immobilized Ni-metal ion affinity chromatography and His6-tag has a strong affinity for nickel ions as shown in the figure below.

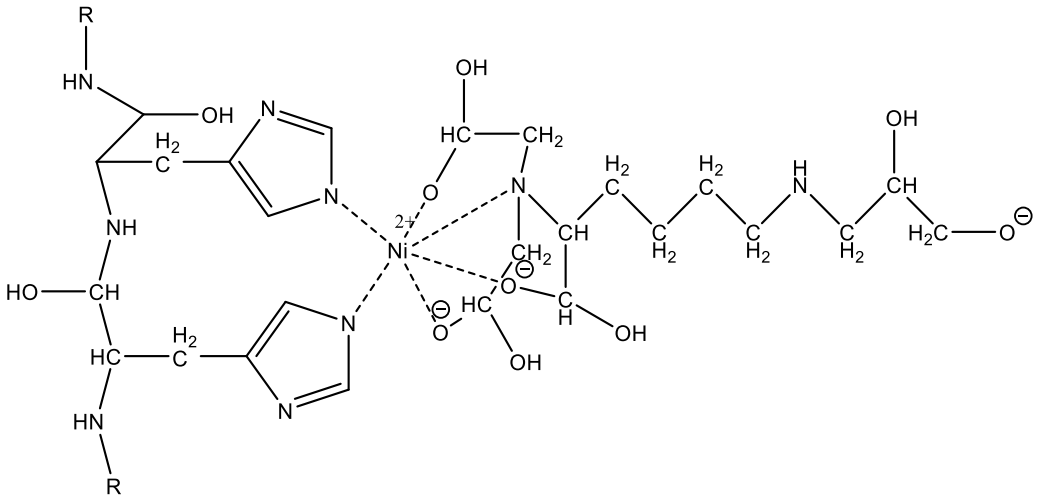


Figure 2.4: Interaction between His6-tag and Ni-NTA matrix

c) SDS – PAGE:

- 40% acrylamide/ bisacrylamide aqueous solution

- **10% SDS solution:** Mix 10 g Sodium Dodecyl Sulfate with 75 ml dd H_2O, then further dilute to a final volume of 100 ml.

- **1.5 M Tris-HCl buffer (pH 8.8):** Dilute 36.33 g Tris base to 150 ml dd H_2O. Adjust the pH to 8.8 by adding dropwise 0.1 M HCl to the solution. The final volume should be reached to 200 ml with dd H_2O and stored at 4^oC.

- **0.5 M Tris-HCl buffer (pH 6.8):** 6.055 g Tris base was added to 70 ml dd H_2O. Then, 0.1 M HCl was added dropwise to adjust the pH to 6.8. The solution was finally diluted to a total volume of 100 ml by dd H_2O and stored at 4^oC.

- **5x sample loading buffer:** 21.5 ml glycerol, 15 ml of 0.5 M Tris-HCl (pH = 6.8) solution and 10 ml of 10% SDS solution was mixed well with 3.5 ml of 0.1% Bromphenol Blue solution, dd H_2O was added to 50 ml to reach the final volume.
- **10x running buffer:** Mix 30.3 g Trizma base, 144.0 g glycine and 10.0 g SDS in 750 mL dd H_2O, adjust pH by 6M HCl, then add dd H_2O up to 1L. Finally, dilute to 1 x running buffer.
- **10% ammonium persulfate (APS) solution:** 0.1 g Ammonium Persulfate was dissolved in 1 ml dd H_2O and stored at 4^oC.
- TEMED
- **Comassive Blue Staining solution:** Mix well 50 ml of Methanol, 10 ml of acetic acid and 0.1 ml Comassive Blue in 39.9 ml dd H_2O.
- **Destaining solution:** 10 ml Methanol and 10 ml Acetic acid was mixed well in 80 ml dd H_2O.

2. Procedure:

a) <u>Overexpression of T7-RNAP:</u>

After incubating the Luria agar medium for 24 hours at 37^oC (the preparation was described above), several small and circular cell colonies were observed. One or two colonies were then picked up and put into 2L of LB solution containing 100 µg/ml ampicillin. Then the cell culture was let to proliferate at 37^oC in a shaker incubator until the A_{600} of the culture reached 0.4 – 0.6 by taking the absorbance at 600 nm every 2 hours, the blank is LB medium. The cultures would be induced for the overexpression of T7-RNA polymerase by adding 100 µM of IPTG, and shaken at 37^oC for an additional 3 hours. Finally, the solutions were centrifuged at 10000 X for

10 - 15 minutes at 0^{o}C. The cell pellets were collected and stored at -80^{o}C for the purification step.

b) <u>Purification of T7-RNAP:</u>

The cell pellets were thawed at room temperature and resuspended in 25 ml of binding buffer. Next, the cells were lysed by sonication for 4 rounds of 15 seconds each at the maximum power recommended for the microtip probe to break the bacterial cell wall and leave us their protein, DNA, and RNA. The cell debris and unlysed cells will be removed by centrifugation at 18,000 X g for 30 minutes at 4^{o}C. The supernatant as the clear lysate was transferred to a fresh 50 mL centrifuge tubes and 20 µL this aliquot was taken for SDS-PAGE analysis later.

Preparing Ni-NTA resin for T7-RNA Polymerase purification:

1. Invert the bottle containing the Ni-NTA Agarose to resuspend all of the ingredients.
2. Pour about 5 mL of resin beads into a 25 mL centrifuge tube, then centrifuge the column at 1000 rpm for 5 min. The supernatant was removed from the resin.
3. Add about 10 mL of dd H_2O to the resin, invert the column, centrifuge at the same rate as in step 2, and then remove the supernatant from the resin.
4. Add 10 mL of binding buffer to the resin and repeat step 3.
5. Repeat step 4 then step 3 for other 2 times.

Now, clear lysate was then bound to this 5 mL of resin with binding buffer, gently rotate for 30 minutes, centrifuge for 5 minutes at 4^{o}C and 20µL aliquot was taken from the supernatant which was discarded for gel analysis. Next, T7-RNAP was washed four times with washing buffer containing 10 mM imidazole at room temperature. Each washing step involved gently pelleting the resin in a clinical centrifuge, discarding the buffer and 20 µL aliquots were kept after each washing step. Finally, resuspending the column of enzyme bound resin in elution

buffer containing 100 mM imidazole to elute our interested protein. Imidazole has a structure with a side chain similar to histidine amino acid and this can bind to the Ni^{2+} ion in immobilized metal affinity chromatography. Thus, the purpose of adding imidazole to the column is to compete with the His-tagged recombinant protein and the protein is eluted. If higher concentration of imidazole is added, more protein will be collected. Now, the pure T7-RNAP was collected.

c) <u>SDS-PAGE Electrophoresis:</u>

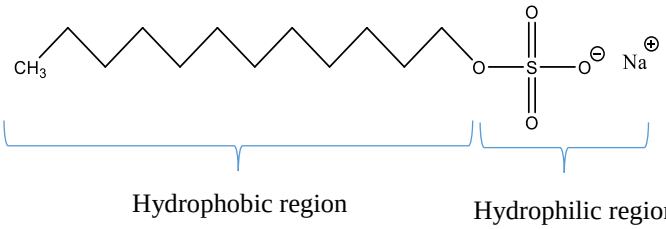


Figure 2.5: Sodium Dodecyl Sulfate

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a technique for separating protein based on the ability to move through the electrical current. Since a protein will have an N-terminus with a positive charge and a C-terminus with a negative charge, by adding SDS detergent, secondary and tertiary of protein structures will be removed and leave the protein as polypeptide chain. The SDS will coat the protein and confer the same negative electrical charge across all the protein polypeptide chain from that protein can migrate through the gel. An SDS-PAGE gel will have 2 components: resolving gel solution (pH = 8.8) with 6 – 10% gel will be placed at the bottom, and stacking gel solution (pH = 6.8) will be on the top of the gel. SDS-PAGE separates proteins according to their molecular weight, based on their differential rates of migration through a gel (the image will be shown in the result and discussion sections).

Before running this electrophoresis, an appropriate size of protein has to be chosen. Different percentage of Acrylamide or Bisacrylamide using should correspond to the size of the protein.

The size of protein (kDa)	%A/B
36 - 205	5%
24 - 205	7.5%
14 - 205	10%
14 - 66	12.5%
10 - 45	15%

Table 2.1: Concentration of Polyacrylamide based on the size of protein

(http://www.proteinchemist.com/tutorial/sdspage.html)

T7 RNA polymerase has a size equal to 98 kD, we choose a 10% Acrylamide gel.

Separating gel (10% A/B) preparation: Mix well 22 ml of 40% (w/v) Acrylamide stock solution, 12.5 ml of 1.5 M Tris-HCl (pH = 8.8) containing 0.4% SDS solution in a small filtering flask, then add dd H_2O up to 50 mL. To run the gel, take out 5 mL of this separating gel solution to another filtering flask, add 80 µl of 10% APS and 8 µl of TEMED to the solution, then pour into between two glass plates and 0.5 mL saturated isoamyl alcohol was added on the top of the gel. The solution will be polymerized for about 30 mins at room temperature. After it became polymerization, pour off isoamyl alcohol, wash the gel with dd H_2O for 3 times and left it dry for about 5 to 7 mins.

Stacking gel (4% A/B) preparation: Mix 5.6 ml of 40% (w/v) Acrylamide stock solution, 7.5 ml of 0.5 M Tris-HCl (pH = 6.8) containing 0.4% SDS solution in a small filtering flask, then add dd H_2O up to 30 mL. To run the gel, take out 3 mL of this stacking gel solution to another filtering flask, add 60 µl of 10% APS and 6 µl of TEMED to the solution. The gel will be slowly pipetted into the glass plates to completely cover the top of 10% stacking gel. Attach the gel comb to create 10 wells and allow to polymerize for another 15 mins at room temperature.

Loading sample: The samples must be completely denatured before running on the SDS gel. To do this, a mixture of 10 µL of 5x loading buffer and 20 µL of sample, incubate at 100ºC for 5 mins, then cool it on ice before loading on the gel. The samples will be loaded and run in 1 x electrophoresis running buffer at the current of 40 mA until the bromphenol blue completely out of the gel.

Staining, Distaining, and Fixing: After finishing running, the gel was stained in Comassive Blue Staining solution for 1.5 hours on the shaker at low shaking force. Then the gel was put into distaining and fixing solution for 30 mins and finally took a picture of the gel.

3. *In vitro* RNA transcription reaction and assembly of RNA nanoparticles:

a) Prepare for transcription reaction:

Equipment needed:

1. Sets of micropipettes 20-100 µL and 2-20 µL
2. Microcentrifuge tubes
3. Water bath set to 37ºC

Solutions needed:

1. 5 x transcription buffer (400 mM HEPES – KOH pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine and 200 mM DTT)
2. 25 mM rNTPs
3. 100 mM DTT
4. Purified DNA template
5. Home-made T7 RNA Polymerase

To set up RNA transcription, reaction mixtures should be prepared at room temperature as below:

Reagents	Volume
Deionized water	5 µL
5 x transcription buffer	10 µL
25 mM rNTPs	10 µL
100 mM DTT	5 µL
DNA template	10 µL
T7 RNA Polymerase	10 µL
V_{tot} = 50 µL	

- The solution will be incubated at 37^{o}C for 6 hours.
- After 6 hours, take out the sample from the incubator, terminate the transcription reaction by adding 1 µL DNase I, incubate for 30 minutes to hydrolyze DNA primers.

b) RNA purification using 8M UREA-PAGE:

RNA molecules synthesized by *in vitro* transcription are purified to remove any remaining RNA polymerase, DNA template, and unused nucleotides. In this step, we are going to purify RNA samples and evaluate their purity using denaturing polyacrylamide gel electrophoresis (UREA-PAGE). RNAs fold into stable and complex structures, therefore, RNA molecules have to be denatured to separate them by molecular weight. Denaturation is achieved using urea will compete with nucleobases for H-bonding thereby unfolding the RNA molecule. Denaturing PAGE is similar to agarose gel electrophoresis of DNAs. Polyacrylamide forms similar net-like porous structure as agarose, but here the pore size is significantly smaller accommodating separation of smaller nucleic acid. RNAs will migrate in the medium because of their negative to the positive electrode. Denaturing PAGE will separate RNA molecules by their molecular weight and produce the cleanest RNA.

Equipment needed:

1. PAGE apparatus
2. Power supply to run urea PAGE
3. TLC plate
4. UV light

Solution needed:

1. RNA samples
2. Tris-borate-EDTA (TBE) buffer, pH = 8.0; 1× buffer composition: 89 mM Tris base, 86 mM boric acid, and 2 mM EDTA.
3. Urea 8 M in 20 mM TBE buffer pH = 8.0.
4. Denaturing polyacrylamide; stock 20 % solution composition: Acrylamide/bis-acrylamide (29:1), 8 M urea, 1× TBE buffer.
5. 8M urea loading dye (8M urea, 0.5 x TBE, 1% bromophenol blue)
6. 10% Ammonium persulfate (APS)
7. TEMED

Making 10% Denaturing PAGE gel:

1. Assemble gel plates and check them for leaking.
2. Urea gel solution mix (15 % acrylamide, 1 x TBE, 29:1 acrylamide:bis-acrylamide, 8 M urea) into a centrifuge tube.
3. Add 70 µL 10% APS and 7 µL TEMED to initiate polymerization.
4. Mix well and pour in between glass plates. Place in comb. It should be done quickly and will take about 20 minutes for the gel to be polymerized.
5. Once gel is polymerized assemble gel gasket.

6. Pour 1 x TBE running buffer into gasket.
7. Wash wells.

Prepare samples and run the gel:

1. Add the same amount of 8M urea loading buffer to the transcription solution and mix well.
2. Load the total amount of samples onto the gel.
3. Run the gel in 1× TBE buffer at 120 V until the bands reach the bottom of the glass plates.
4. Place the gel on a TLC plate and excise the RNA band under UV light.
5. Place the pieces of the gel into the centrifuge tubes and crush the gel by using the pipet tip.
6. Extract RNA from gel slice in 1 ml of RNA elution buffer (0.5 M NH_4OAc, 10 mM EDTA, 0.1 % SDS) and store at 4^{o}C for 2 hours or overnight.

Precipitate RNA:

1. Centrifuge the mixture (gel + buffer) for 20 minutes, collect 400 µL solution and transfer to the centrifuge tube.
2. Add 1000 µL 100% cold EtOH (1:2.5 volume ratio) and 10 µL 3 M sodium acetate (1:10 volume ratio) to the solution, vortex and place at -20^{o}C for 1 hour.
3. Centrifuge for 20 minutes, discard liquid and keep the pellet.
4. Wash with 75% cold EtOH, centrifuge for 10 mins, dry under vacuum, and redissolve in 30 µL dd H_2O.
5. Measure RNA concentration and store all samples at −20 °C.

c) RNA concentration calculation:

RNA concentration is determined in a similar manner as DNA concentration. As DNA, RNAs also absorb at 260 nm because of the heterocyclic nucleobases. To calculate RNA concentration, we are going to use the Beer-Lambert law:

$$\mathbf{A\ (@260\ nm) = \varepsilon * c * 1}$$

Where l = 1 cm, c is the concentration in mole/L and ε is the wavelength-dependent molar absorptivity coefficient with units of $M^{-1}cm^{-1}$.

IV. Anneal RNA strands to form RNA nanoparticle:

1. RNA nanoparticle assembly:

1. Mix RNA strands corresponding to triangle, square, and pentagon (1 μM final concentration) in 1× TMS, and bring final volume to 10 μL with dd H_2O (see Table 3).
2. The mixture will be heated at 80°C for 5 minutes and slowly cool down at 4°C for about 20 minutes.

	1	2	3	Tri	5	Sqr	7	Pent	9	Hex
10x TMS (µL)	1	1	1	1	1	1	1	1	1	1
10µM rT1 (µL)				1						
10µM rT2 (µL)			1	1	1	1	1	1	1	1
10µM rT3 (µL)	1	1	1	1	1	1	1	1	1	1
10µM rT4 (µL)		1	1	1	1	1	1	1	1	1
10µM rS5 (µL)					1	1	1	1	1	1
10µM rS1 (µL)						1				
10µM rP6 (µL)							1	1	1	1
10µM rP1 (µL)								1		
10µM rH7 (µL)									1	1
10µM rH1 (µL)										1
dd H_2O (µL)	8	7	6	5	5	4	4	3	3	3

Table 2.2: Table of RNA polygons assembling setup

2. RNA nanoparticle assembly evaluation by native PAGE:

While Denaturing PAGE is used to denature and separate RNA molecules based on their molecular weight, here Native PAGE will be run under non-denaturing condition in a purpose to maintain the natural structure of RNA nanoparticles.[49]

Equipment needed:

1. PAGE apparatus
2. Power supply to run urea PAGE

Solution needed:

1. RNA nanoparticle samples

2. Low molecular weight ladder
3. Tris-borate-magnesium (TBM) buffer, pH = 8.0; 1× buffer composition: 89 mM Tris base, 86 mM boric acid, and 5 mM $MgCl_2$.
4. 40% acrylamide stock
5. 6 x Native loading dye (600 mM Tris-HCl, 50% Glycerol, 0.02% Bromophenol blue).
6. 10% EtBr
7. 10% Ammonium persulfate (APS)
8. TEMED

Making 6% Native PAGE gel:

1. Assemble gel plates and check them for leaking.
2. Urea gel solution mix (6 % acrylamide, 1 x TBM, 40% acrylamide stock) into a centrifuge tube.
3. Add 80 µL 10% APS and 8 µL TEMED to initiate polymerization.
4. Mix well and pour in between glass plates. Place in comb. Watch out, this is quick and it will take about 20 minutes for the gel to be polymerized.
5. Once gel is polymerized assemble gel gasket.
6. Pour 1 x TBM running buffer into gasket.
7. Wash wells.

Prepare samples and run the gel:

1. Load 2 µL of the samples and 2 µL molecular weight ladder (with 2 µL loading dye) onto the gel.
2. Run the gel in 1× TBM buffer at 90 V for about 30 minutes.

3. Place the gel into staining solution (15 mL TBE buffer or dd water and 2 µL EtBr) for 10 minutes.
4. Take picture of gel using a gel documentation system.

3. RNA nanoparticles purification:

1. Set up a 6% native PAGE as shown in section 2.4.2.
2. Add the same amount of 6X native loading buffer to the RNA nanoparticle solution and mix well.
3. Load the total amount of samples onto the gel.
4. Run the gel in 1× TBM buffer at 90 V until the bands reach the bottom of the glass plates.
5. Place the gel on a TLC plate and excise the RNA band under UV light.
7. Place the pieces of the gel into the centrifuge tubes but do not crush the gel at this time.
8. Extract RNA from gel slice in 1 ml of RNA elution buffer and 10 mM $MgCl_2$
9. Store at 4^{o}C overnight.

Precipitate RNA nanoparticle:

Follow the steps to precipitate RNA samples, but RNA nanoparticles will rehydrate in TMS buffer instead of dd H_2O.

V. <u>Determine the melting temperature of RNA nanoparticle:</u>

1. UV melting experiment:

UV melting is a method to unwind double strands of nucleic acid by heating. When the temperature is increased, the double strands of nucleic acids will begin to dissociate and when the temperature reaches a point at which 50% of the double strands is denatured will be known

as a melting point. Absorbance versus temperature curves can provide information on the thermal stability of DNA or RNA quadruplexes.[50]

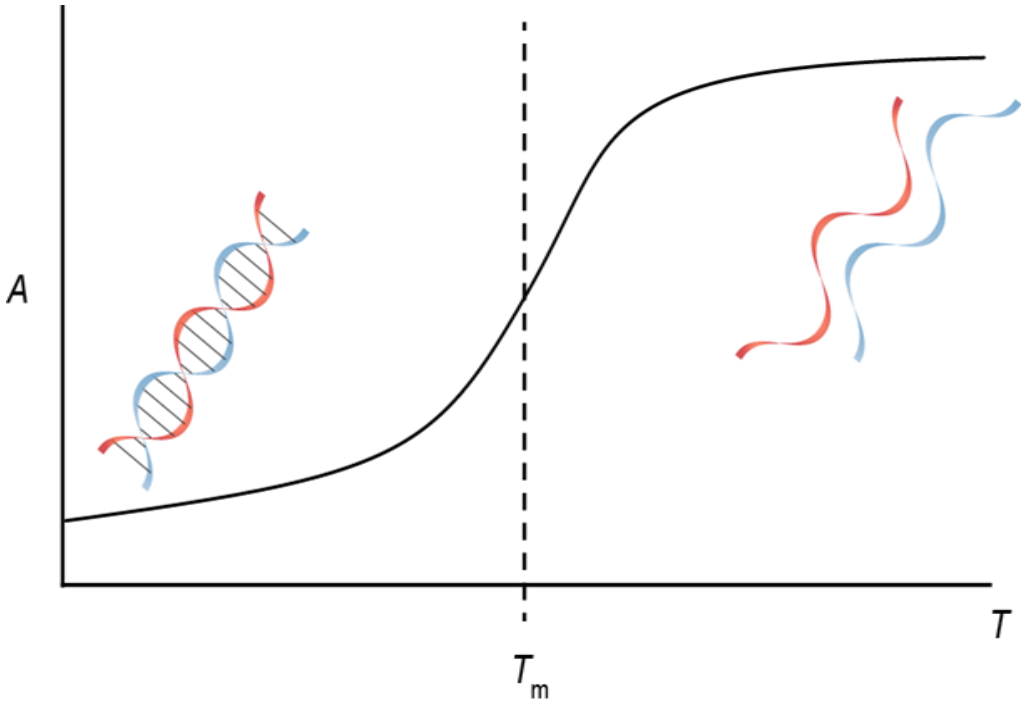


Figure 2.6: The profile of UV absorbance versus temperature is called a melting curve; the midpoint of the transition is defined as the melting temperature, Tm.

2. Materials:

- RNA nanoparticle
- 10 x Sodium CaCodylate buffer pH = 7
- 3 M Sodium Chloride salt
- 2 Quartz cuvettes and caps (for blank and samples)
- UV-visible spectrophotometer equipped with a cell changer device
- Temperature control device
- Fan dryer
- Computer attached to spectrophotometer

3. Prepare RNA polygons stock solution and determine the absorbance:

- Make 1 x buffer containing 1 x sodium cacodylate and 0.1 M NaCl from 400 mM sodium cacodylate and 3M NaCl.
- Prepare 1 µM RNA polygons solution in 1 x sodium cacodylate.
- Check the absorbance and the concentration of RNA polygons by using Nanodrop 1000 Spectrophotometer at 260 nm. The absorbance should be between 0.5 and 1.

4. Running UV-visible spectrophotometer to determine T_M of RNA polygons:

- Turn on spectrometer and choose Thermal denaturation program.
- Blank the spectrometer with 1 x Sodium Cacodylate buffer only.
- Change the absorbance in the range from 260 nm to 260 nm since the nucleic acid has an absorbance at 260 nm.
- Set the temperature to run from 20^{o}C to 90^{o}C for the first cycle, then from 90^{o}C to 20^{o}C for the second cycle with increment is 1^{o}C and the hold time is 0.02 min.
- Slow heating of the nanoparticles causes the unwinding of the ordered helical structure into the two single-stranded constituents. This can be seen as a sigmoidal curve of increasing UV absorption. The mid-point, corresponding to the precise melting temperature (Tm) of the duplex, is indicated.

VI. <u>Evaluation stability of nanoparticles in Fetal Bovine Serum:</u>

1. Fetal Bovine Serum (FBS) stability experiment:

FBS is the blood fraction remaining after the natural coagulation of blood, followed by centrifugation to remove any remaining red blood cells. Potent RNase activities are found in the serum, and RNase is a type of nuclease that will catalyze the degradation of RNA into smaller

components. Therefore, which RNA nanostructure is more stable in serum, it will be better for treatment in medicine.[51]

2. Samples preparation for serum:

a) Checking stability of each polygon in serum:

Make 10 µL of 1 µM of RNA, DNA and hybrids (RNA/DNA-center and DNA/RNA-center) nanoparticles: Triangle, Square, Pentagon, and Hexagon from 10 µM of each RNA and DNA strands. Then mix 4 µL of each polygon with 4 µL of 10% FBS (Sigma-Aldrich™) and incubate 1h at 37°C.

	Tri	**Sqr**	**Pent**	**Hex**
10x TMS (µL)	1	1	1	1
10 µM dT1 (µL)	1			
10 µM dT2 (µL)	1	1	1	1
10 µM dT3 (µL)	1	1	1	1
10 µM dT4 (µL)	1	1	1	1
10 µM dS1 (µL)		1		
10 µM dS5 (µL)		1	1	1
10 µM dP1 (µL)			1	
10 µM dP6 (µL)			1	1
10 µM dH1 (µL)				1
10 µM dH7 (µL)				1
dd H_2O (µL)	5	4	3	2

Table 2.3: Table of assembling DNA polygons

	Tri	Sqr	Pent	Hex
10x TMS (µL)	1	1	1	1
10 µM dT1 (µL)	1			
10 µM dS1 (µL)		1		
10 µM dP1 (µL)			1	
10 µM dH1 (µL)				1
10 µM rT2 (µL)	1	1	1	1
10 µM rT3 (µL)	1	1	1	1
10 µM rT4 (µL)	1	1	1	1
10 µM rS5 (µL)		1	1	1
10 µM rP6 (µL)			1	1
10 µM rH7 (µL)				1
dd H_2O (µL)	5	4	3	2

Table 2.4: Table of assembling RNA/DNA-center hybrid polygons

	Tri	Sqr	Pent	Hex
10x TMS (µL)	1	1	1	1
10 µM dT2 (µL)	1	1	1	1
10 µM dT3 (µL)	1	1	1	1
10 µM dT4 (µL)	1	1	1	1
10 µM dS5 (µL)		1	1	1
10 µM dP6 (µL)			1	1
10 µM dH7 (µL)				1
10 µM rT1 (µL)	1			
10 µM rS1 (µL)		1		
10 µM rP1 (µL)			1	
10 µM rH1 (µL)				1
dd H_2O (µL)	5	4	3	2

Table 2.5: Table of assembling DNA/RNA-center hybrid polygons

b) Stability of triangular nanoparticles in serum:

2% FBS degradation Protocol steps:

1. Assemble 1µM triangle in 50 uL in 1x TMS buffer.
2. Prepare 7 centrifuge tubes and label them as 0, 1, 5, 15, 25, 40 and 60. Add 2 µl of 6x loading dye to each one.
3. Start program transcription of PCR thermocycler so you have the temperature ready.
4. Take 39.8 µL of the assembled triangle and add 0.8 µL of 100% FBS, adjust pipettor to 5 µL and mix by pipetting up and down (The resulting FBS concentration = 2%).
5. Take 5 µL from the resulting mixture and transfer into the test tube labeled “0” containing 2 µL of 6x native dye. Place it into the freezer (this is time 0).

6. Put the rest into 37ºC (PCR thermocycler) and after certain time intervals take 5 µL aliquots and transfer into prepared centrifuge tube containing 2 µL 6x native loading dye. Do not forget place into freezer.

In parallel:

Incubate 2% FBS in thermocycler @ 37ºC for 1 h.

Incubate triangle only @ 37ºC for 1 h.

VII. Functionalization nanoparticles with siRNA:

1. Designing RNA strands carrying siRNA

The small interfering RNA or siRNA is a specific double-stranded RNA of approximate 25 nucleotides conjugated at 5' end of each external strand of each nanoparticle:

siRNA Sense strand: 5' - CGGUGGUGCA GAUGAACUUC AGGGU - 3'[52]

DNA-sense-Alexa546 (for in vitro transfection experiments):

5'-/5AlexF546N/aaTGACCCTGAAGTTCATCTGCACCACCG[52]

(See Appendix for functionalized RNA strands)

2. Assembling nanoparticles carrying siRNA:

Make 60 µL of 1 µM RNA/DNA center hybrid triangle nanoparticle conjugate with siRNA and DNA Alexa:

Tubes / Reagents	#1	#2
10 x TMS (µL)	6	6
10 µM dT1 (µL)	6	6
10 µM rT2 (µL)	6	6
10 µM rT3 (µL)	6	6
10 µM rT4 (µL)	6	6
10 µM siRNA (µL)	0	2.4
10 µM DNA Alexa (µL)	2.4	0
dd H_2O	27.6	27.6

Table 2.6: Table of assembling RNA/DNA hybrid triangle nanoparticle conjugate with siRNA and DNA Alexa

VIII. Atomic Force Microscopy sample preparation:

A freshly cleaved mica (Ted Pella) surface was pre-treated with 1 mM $NiCl_2$, air dried. Then the 50 nM purified RNA triangle nanoparticles were deposited to the center of the mica and ready for imaging on AFM 5500 (Keysight Technologies).

Chapter 3: Results and Discussion

I. PCR reaction optimization:

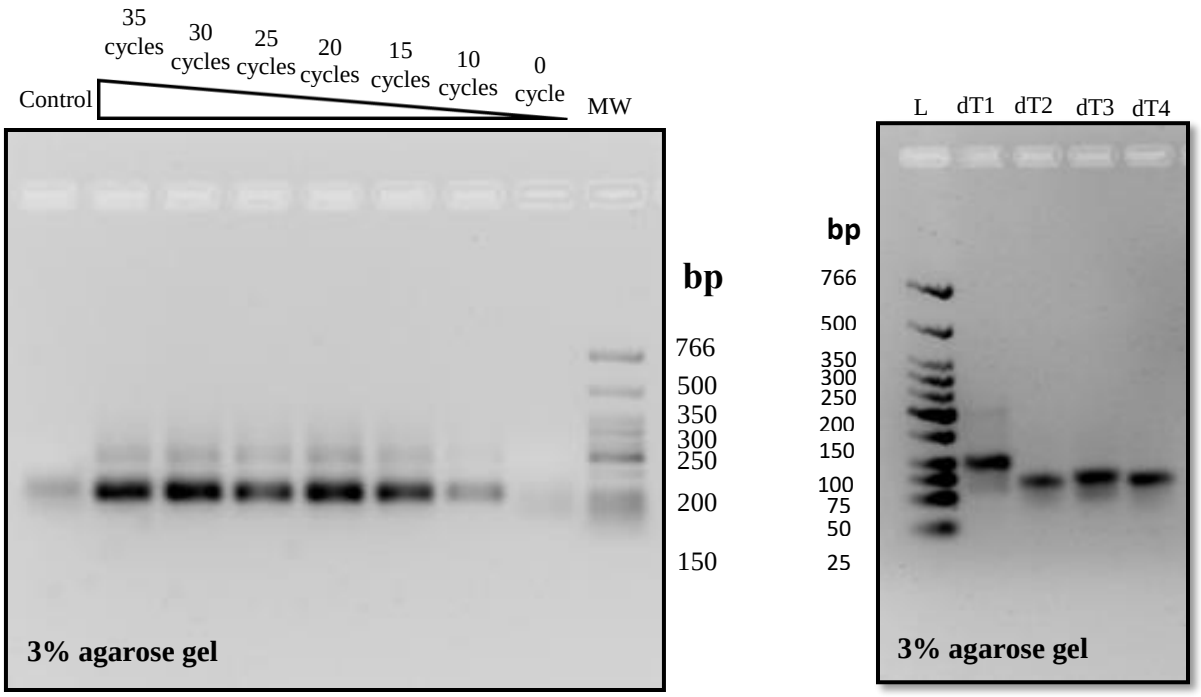


Figure 3.1: PCR reaction optimization (left) of dT1 and PCR products (right) of dT1, dT2, dT3, and dT4

These 3% agarose gels were run after PCR reaction. The left one shows the optimization taken at 5 cycle interval of dT1. Well 1 from the left is the control that was not treated with TAQ enzyme. Without the enzyme, the PCR reaction occurred very slowly, and DNA template was not synthesized, therefore the DNA band did not appear on the gel. Wells 2 to 8 are the aliquots taken after a 5 cycle interval. At 0 cycle, nothing happened since the reaction did not start. After 10 cycles, the DNA template started producing, and the band was more intensive than at 0 cycle. From 15 to 35 cycles, PCR reaction synthesized more DNA. We can recognize this by comparing the intensity of the DNA bands on the gel. The right one is the PCR products of dT1, dT2, dT3 and dT4 that are composition of triangular nanoparticles. On the gel, dT1 is the longest

strand about 150 base pairs and migrates slowest while dT2, dT3, and dT4 have the same size and shorter about 100 base pairs, therefore they migrate faster.

II. Overexpression of T7 RNA Polymerase:

1. SDS – PAGE:

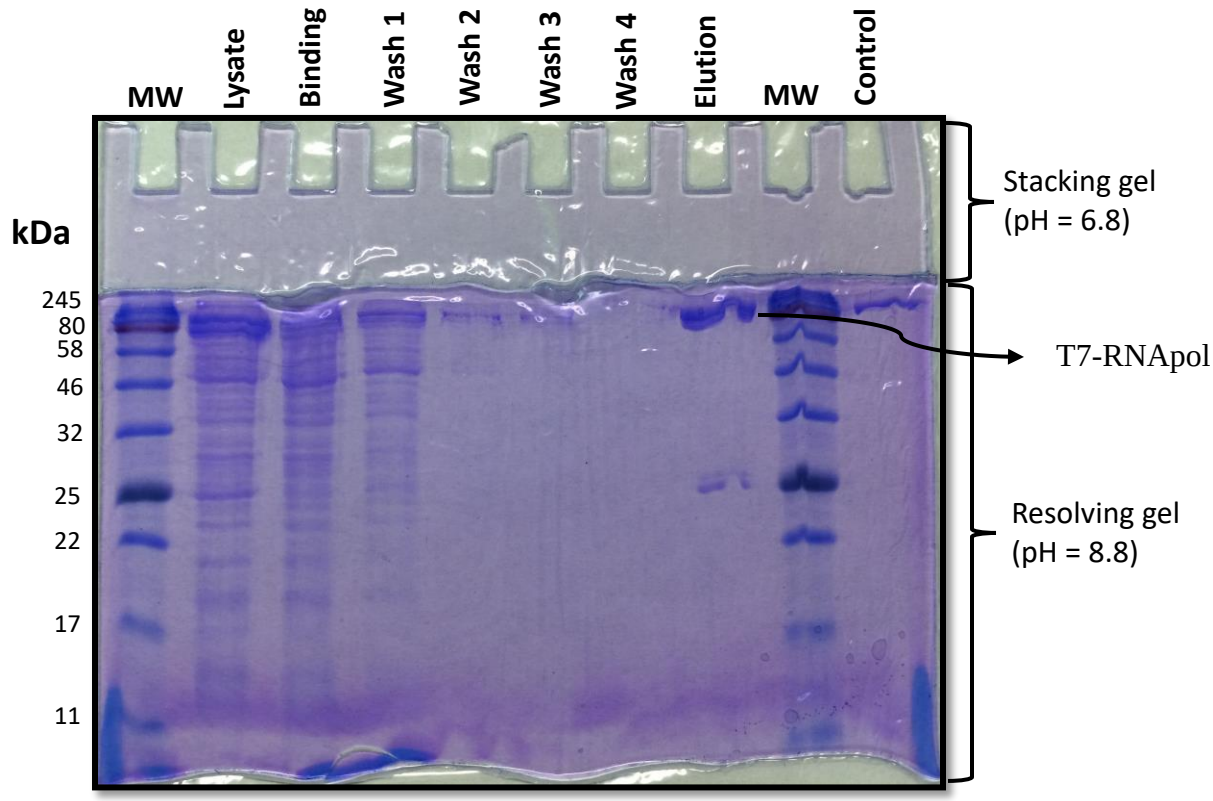


Figure 3.1: SDS – PAGE gel of T7 RNA Polymerase purification

MW – Molecular weight marker.

Lysate - Aliquot consisting of the materials released from cell lysis by sonication.

Binding – Aliquot after binding protein to the resin.

Wash 1, Wash 2, Wash 3, and Wash 4 – Aliquots taken after each washing step.

Elution – Aliquot after elution with 100 mM imidazole solution.

Control – The commercial T7 RNA Polymerase.

The gel has two layers with different functions: the stacking gel on the top is used to equilibrate or concentrate the proteins into one band to make them migrate at the same time in the resolving gel. Then the resolving gel will separate the protein in the sample based on their molecular weight. The lysate and binding aliquots are crude extract and still contain bacterial protein, their DNA, RNA, cell debris and unlysed cells, therefore, there were multiple bands showed up on the gel. When the column was washed four times with washing buffer containing 10 mM imidazole, the resin with bounded enzyme was separated from the washing buffer and the aliquots were taken after each washing step. The amount of the protein band was decreased after the first washing, the second washing, the third washing and almost clear after the final washing indicated that most of the protein now bounded to the resin and the contaminants were eliminated. Finally, the protein of interested was eluted from the column using elution buffer containing 100 mM imidazole, the sample was taken for running the gel. In this step, imidazole competes with the His-tagged to the recombinant protein and the protein is eluted out. The elution buffer contains a higher concentration of imidazole than the binding and washing buffers in a purpose to collect more protein of interested. The protein band in this step is more intensity and unique since there are no more contaminations and it is consistent with the commercial protein about 98 kDa molecular weight.

2. T7 RNA Polymerase activity determination in transcription reaction:

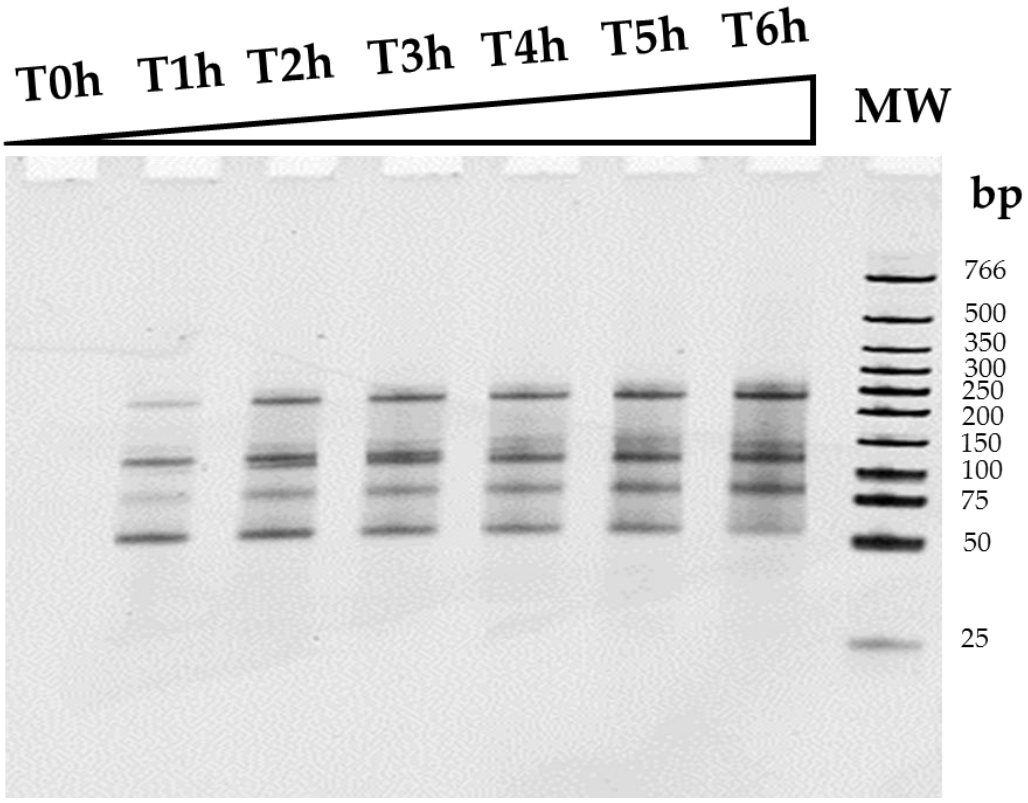


Figure 3.2: Assessment the activity of purified T7 RNA Polymerase

The activity of the T7 RNA Polymerase was tested in a transcription reaction to synthesize rT1 and run on a 10% denaturing PAGE gel. Lane 8 is molecular weight ladder. Wells 1 to 7 are aliquots at 1 hr interval and obtained using the synthesized T7 polymerase as assigned above. Based on this gel, we concluded that the synthesized T7 polymerase could be used for the transcription reaction because rT1 was created after an hour incubation and even more after 6 hours based on the intensity of the bands.

III. RNA transcription:

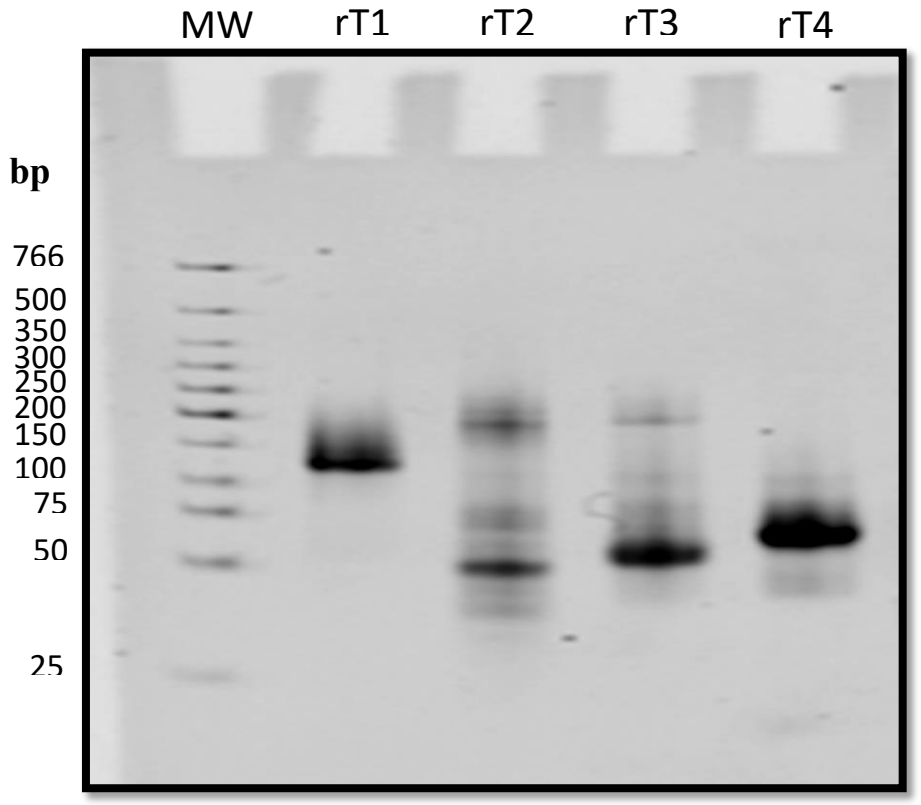


Figure 3.3: Purified RNA products on 10% Denaturing PAGE gel

This 10% Denaturing PAGE gel was run after purifying RNA products. There are four RNA strands synthesized and used to assemble the triangular nanoparticle. Look back to chapter 2, rT1 is the internal and the longest strand while rT2, rT3, and rT4 are the external strands and have the same size to each other. This is consistent with what shows on the gel: rT1 has a molecular weight at about 150 base pair; therefore it migrates slowest, rT2, rT3, and rT4 have a same molecular weight at about 50 base pair and have a same migration. rT2 shows 3 multiple bands just because it should have contamination during the PCR and transcription reactions.

IV. RNA Triangle nanoparticle self-assembly and characterization:

1. RNA triangle “one-pot” self-assembly principle:

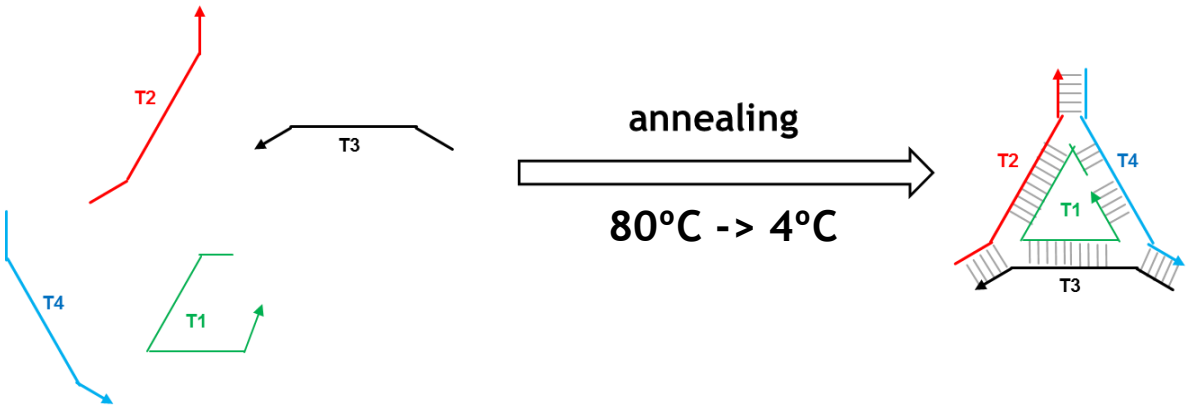


Figure 3.4: Triangle self-assembly scheme

This scheme shows the principle of self-assembly of RNA triangle. As described, RNA triangle is consist of 4 RNA single strands: rT1 (78 nucleotides), rT2, rT3, and rT4 (each has 54 nucleotides). The annealing process will begin with mixing these 4 RNA strands with 1x TMS buffer in a 0.65 mL centrifuge tube, heat the solution to 80^{o}C for 5 minutes, then slowly cool down to 4^{o}C for about 20 minutes. In the result, the external strands will be complimentary with the appropriate nucleotide in the internal strand by Watson – Crick base pairing to create a tetramer. This scheme could be used to create RNA/DNA hybrids as well as DNA triangle nanoparticles. The two hybrid triangles RNA/DNA-center and DNA/RNA-center made from a mixture of RNA and DNA as follows: RNA/DNA-center has dT1, rT2, rT3, rT4 the DNA/RNA-center hybrid is made of rT1, dT2, dT3, and dT4. The DNA triangle has been constructed from DNA strands with dT1 as the central strand, dT2, dT3, and dT4 are external strands. By varying the central strand within RNA triangle or DNA triangle, this will allow us to obtain NPs possessing different physicochemical properties from triangle nanoparticle.

2. Triangles assembly evaluation on NATIVE PAGE:

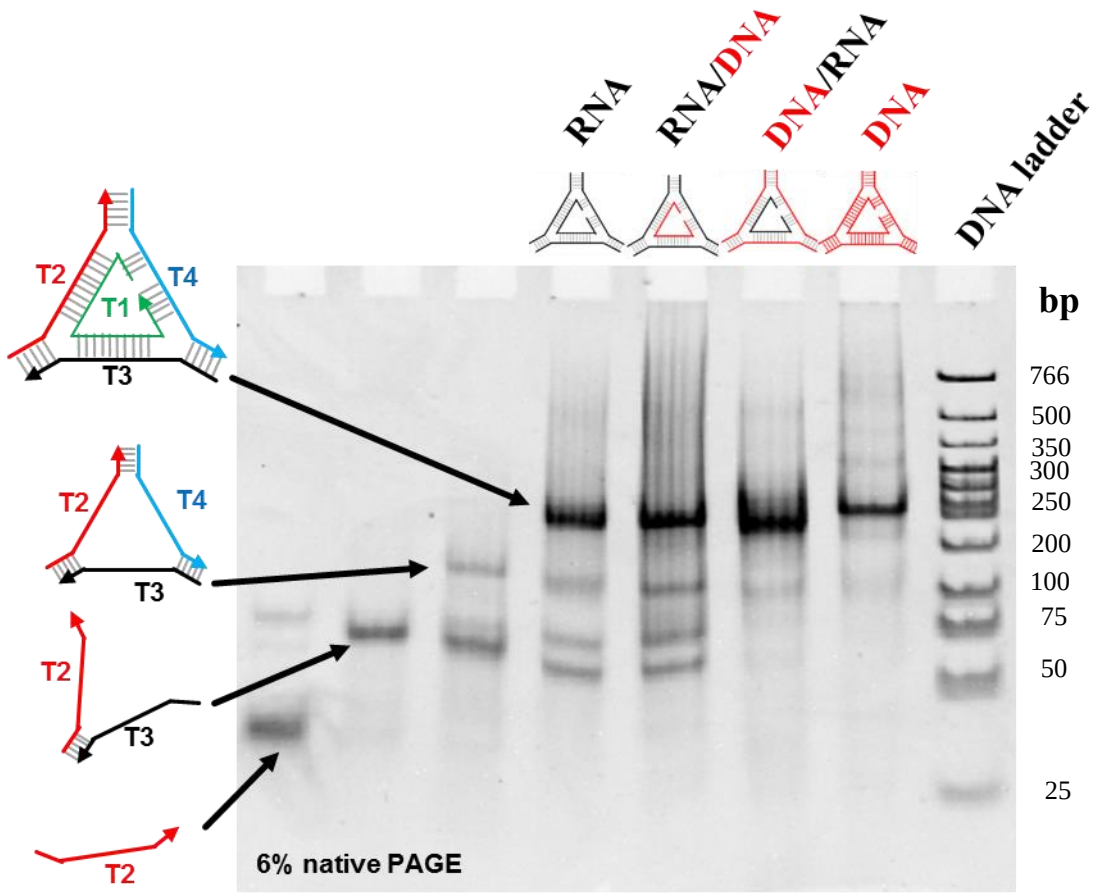


Figure 3.5: Triangle assembly on Native PAGE gel

The 6% Native PAGE gel shows the assembling results of RNA, RNA/DNA hybrids and DNA triangle nanoparticles as explained above. The gel shows the migration based on molecular weight of RNA strands and the nanoparticles. The first well is single strand rT2 called monomer that has a lowest molecular weight at about 40 base pairs and migrates the fastest. The second well is the combination of rT2 and rT3 strands to produce a dimer and has a higher weight of 75 base pairs, therefore, it migrates slower than the monomer. The third well is called trimer where three RNA strands rT2, rT3, and rT4 are annealed together with a molecular weight at 150 base pairs. The fourth to the seventh wells indicate the assembly of RNAs and DNAs strands to create

tetramers. These tetramers all have the same size at 250 base pairs as the largest weight and migrates the slowest. Also, on the gel, DNA tetramer has one intensive band while RNA tetramer has several bands corresponding to trimer and dimer. This indicates DNA will assemble into only one concrete nanostructure while RNA can give alternative nanostructures in a mixture of several strands.

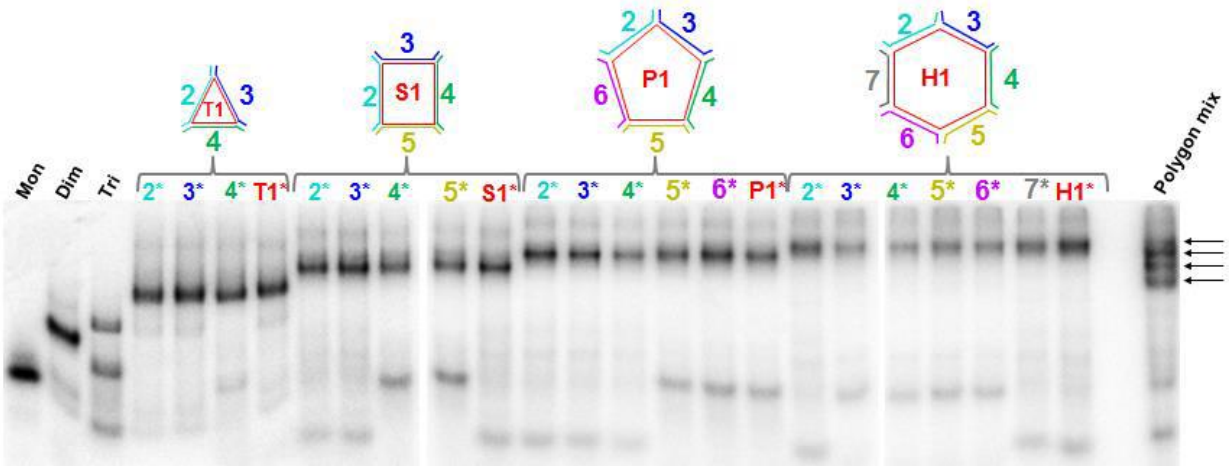


Figure 3.6: RNA self-assembly, assessment on native PAGE using ^{32}P radiolabeling

This 6% native PAGE containing radiolabeled NPs were run in the same condition as non-radioactive. After being dried under vacuum, the gels were exposed to phosphor-imaging screen and scanned using a Storm phosphorimager (Amersham Biosciences) radiolabeled RNA polygons self-assembly. In general, this radiolabeling was used to target the presence of each RNA strand in each of the polygon.

3. RNA triangles assembly evaluation on AFM (atomic force microscope):

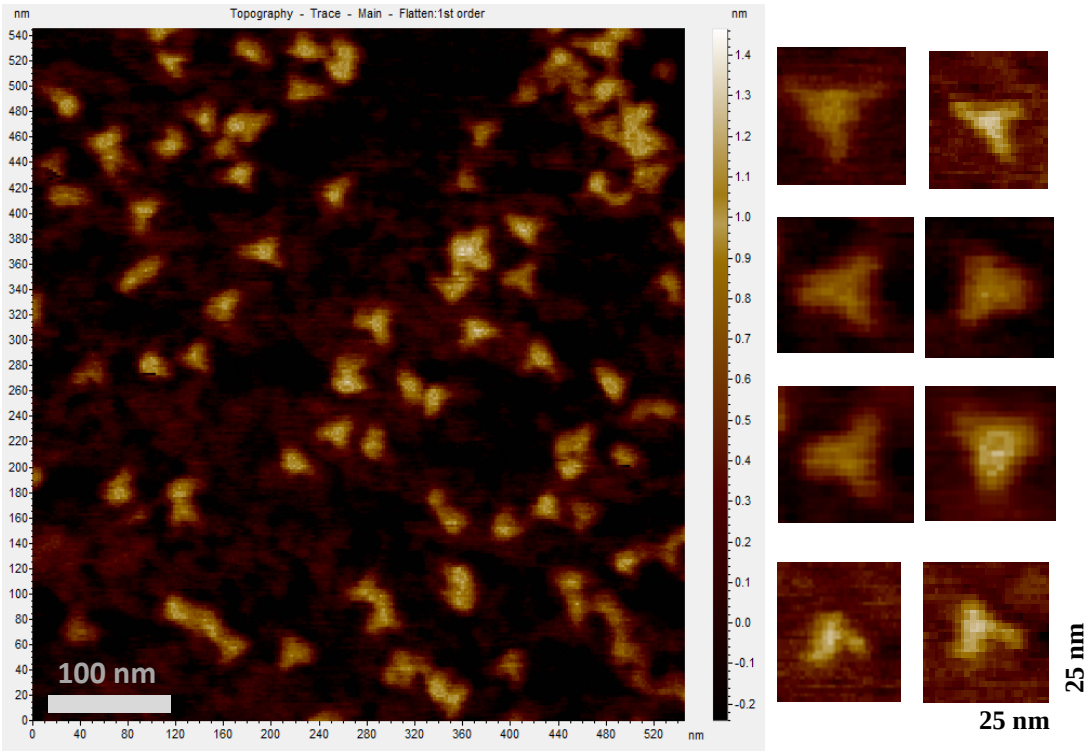


Figure 3.7: RNA triangle AFM images acquired in air at ambient temperature. The AFM images of RNA complexes was done by Mrs. Heather Ramey at BSU.

The evaluation of RNA triangle nanoparticle on native PAGE does not tell us that the nanoparticle we created is a triangular shape. It can be something that is a combination of four RNA single strands. Getting the image on the AFM is one of the ways to check out the shape of this nanoparticle. Atomic force microscopy is the most versatile and powerful microscopy technology for studying samples at nanoscale.[53] Consistent with the theoretical model structures, the AFM images revealed triangle shaped features. The measured average diameter of the triangles was found to be about 16 nm. Statistical analysis of the nanoparticles counted in a 2 x 2 µm mica surface shows that ~ 80 % of them have a triangle or triangle-like shapes.

4. Fine – tunable thermodynamic stabilities of triangle nanoparticle:

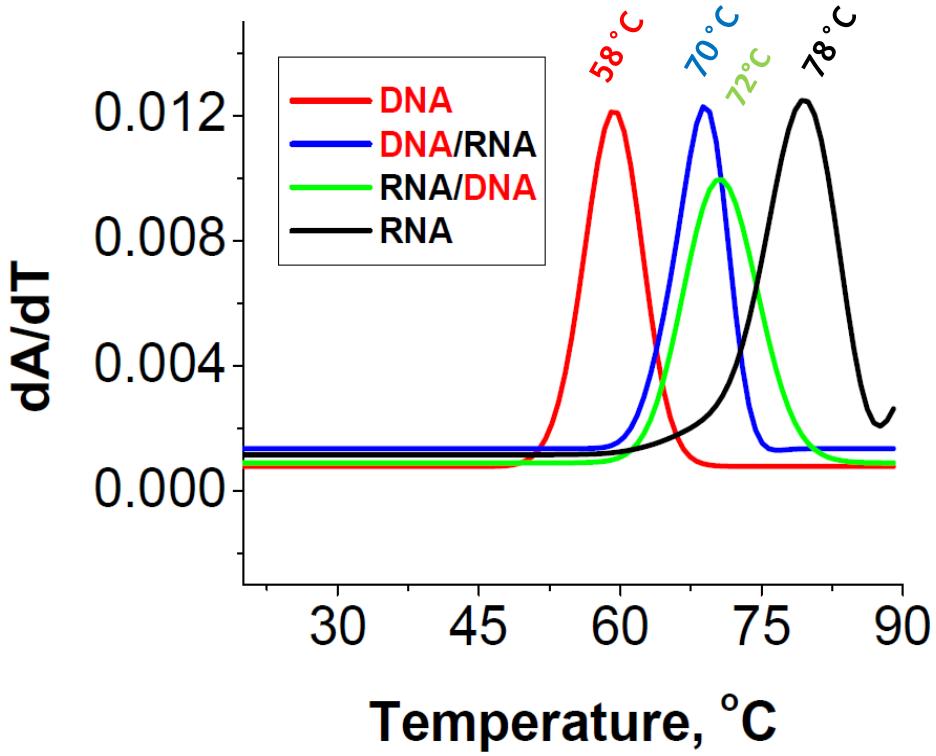


Figure 3.8: 1st derivative showing thermal stabilities of RNA, DNA, and hybrid triangles.

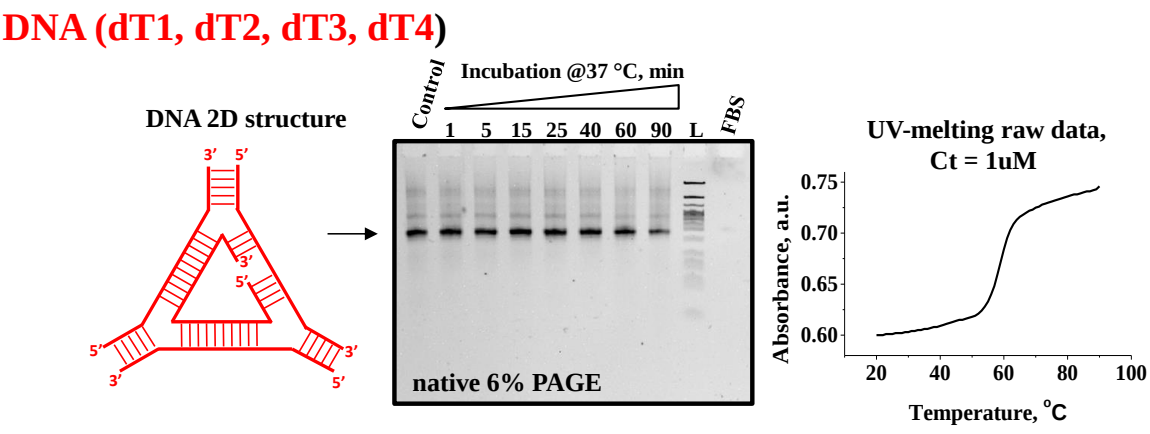
After confirming the triangular nano-scaffold formation, the stability of the nanoparticle was then investigated in solution with respect to temperature. To approach this goal, UV-absorbance of nucleic acid nanoparticles at 260 nm were observed in regularly increasing temperature from 20°C to 90°C known as a UV-melting curve that was described in chapter 2. Figure 3.7 shows a comparison thermal stabilities of RNA, DNA, and two hybrid triangles. The first derivatives of the resulting curves determine the melting temperature (Tm) of each nanoparticle as summarized in the table below:

NP types	Melting temperature (T_M) ^oC
RNA	79.2 ± 0.2
RNA/DNA	71.0 ± 1.5
RNA/DNA	68.8 ± 1.2
DNA	59.5 ± 2.0

Table 3.1: Melting temperature of triangular nanoparticles

Based on this table, RNA nanoparticle has the most thermal stability, followed by two hybrids, and the triangle containing only DNA strands has the least thermal stability. It is very interesting that the transition temperatures vary about 10^{o}C between RNA and hybrids as well as between hybrids and DNA nanoparticles. The difference between RNA-based triangle and DNA triangle is about 19.7^{o}C that can be explained as RNA is in the A-form conformation of the helix and has better base stacking energy compared to the B-form of the DNA. Therefore, when the ratio of RNA and DNA strand is changed in the nanoparticle, the thermal stability can be quite controlled, and this will be the advantage of the system for nanofabrication.

5. **Fine-tunable chemical stabilities of triangular nanoparticles:**



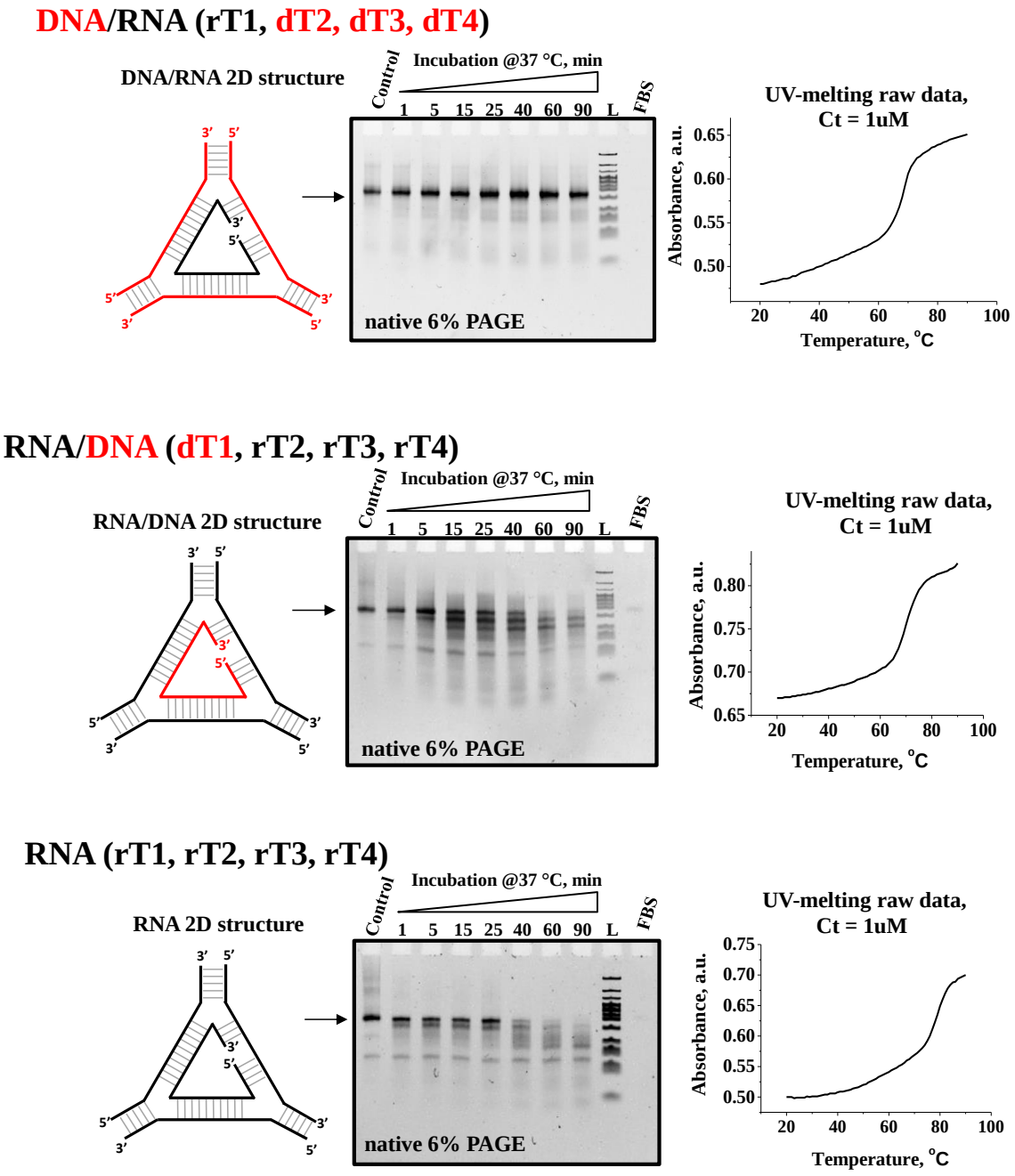


Figure 3.9: Combined raw data of the serum stability and UV-melting studies of DNA, DNA/RNA, RNA/DNA and RNA triangles. Lane FBS corresponds to the sample that contained 2% FBS only incubated for 1.5 h to confirm there is no nucleic acid to interfere with NP bands. Control is the NP only incubated for 90 min.

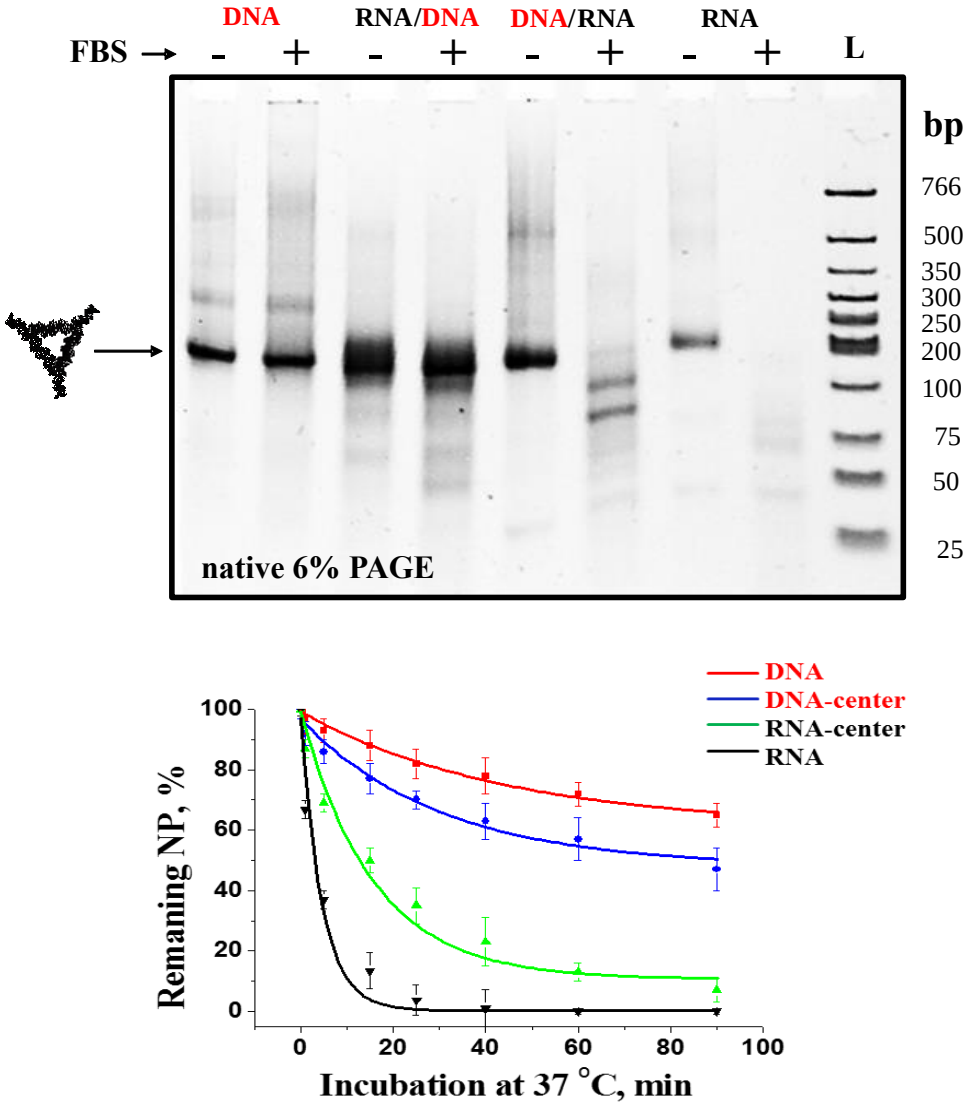


Figure 3.10: Enzymatic stability assay of RNA, DNA, and hybrid triangles after 1h incubation at 37ºC.

There are several methods that can be used to increase the stability of RNA-based nanoparticles *in vivo*. One of the methods is to use 2'-fluoro pyrimidines (2'F-U and 2'F-C) as modified RNA nucleotides during RNA synthesis as mentioned in chapter I. In this research, we evaluated the stability behavior of the triangle nanoparticles in the presence of nuclease in Fetal Bovine Serum (FBS). The triangle nanoparticles were incubated in 2% FBS for 1 hour, and the products were then analyzed on 6% native PAGE. Figure 3.10 demonstrates the degradation of DNA, DNA/RNA, RNA/DNA and RNA-based triangles after 90 min incubation at 37ºC in

absence "-" and presence "+" of 2% (v/v) FBS. The difference in band intensity is due to the different binding affinities of EtBr to DNA and RNA. After visualizing the nucleic acid in EtBr solution, the band qualification from the gel was performed on ImageJ that will allow to determine the remaining fraction of corresponding triangles. The results suggest that the stability of the nanoparticles in FBS is dependent on the composition of the nucleic acid present in each of the triangular complex. The RNA nanoparticles is completely degraded while the RNA/DNA hybrids and DNA nanoparticle are found to be stable. DNA/RNA-center hybrid degrades much faster than RNA/DNA-center hybrid nanoparticle; however, they are still stable overall than the RNA complex.

V. RNA Triangular nanoparticle application in RNA interference:

1. Knowledge about siRNA:

In the body, protein is a complex molecule and play many key roles: structure, function, and regulation of the body's tissues and organs. One of the methods used to treat disease is to inhibit protein by targeting the corresponding mRNAs.[54] In this case, RNA interference is known as one type of RNA molecule that can regulate gene expression through destruction of specific mRNA molecule and therefore, it can prevent an mRNA from producing protein.[55] There are two types of RNAi: small interfering RNA (siRNA) and micro RNA (miRNA). Here, we are going to investigate the use of triangle nanoparticle functionalized with siRNA for delivery into the cell; this way may improve the loading of RISC (RNA-induced silence complex) presented in cytoplasm.[52]

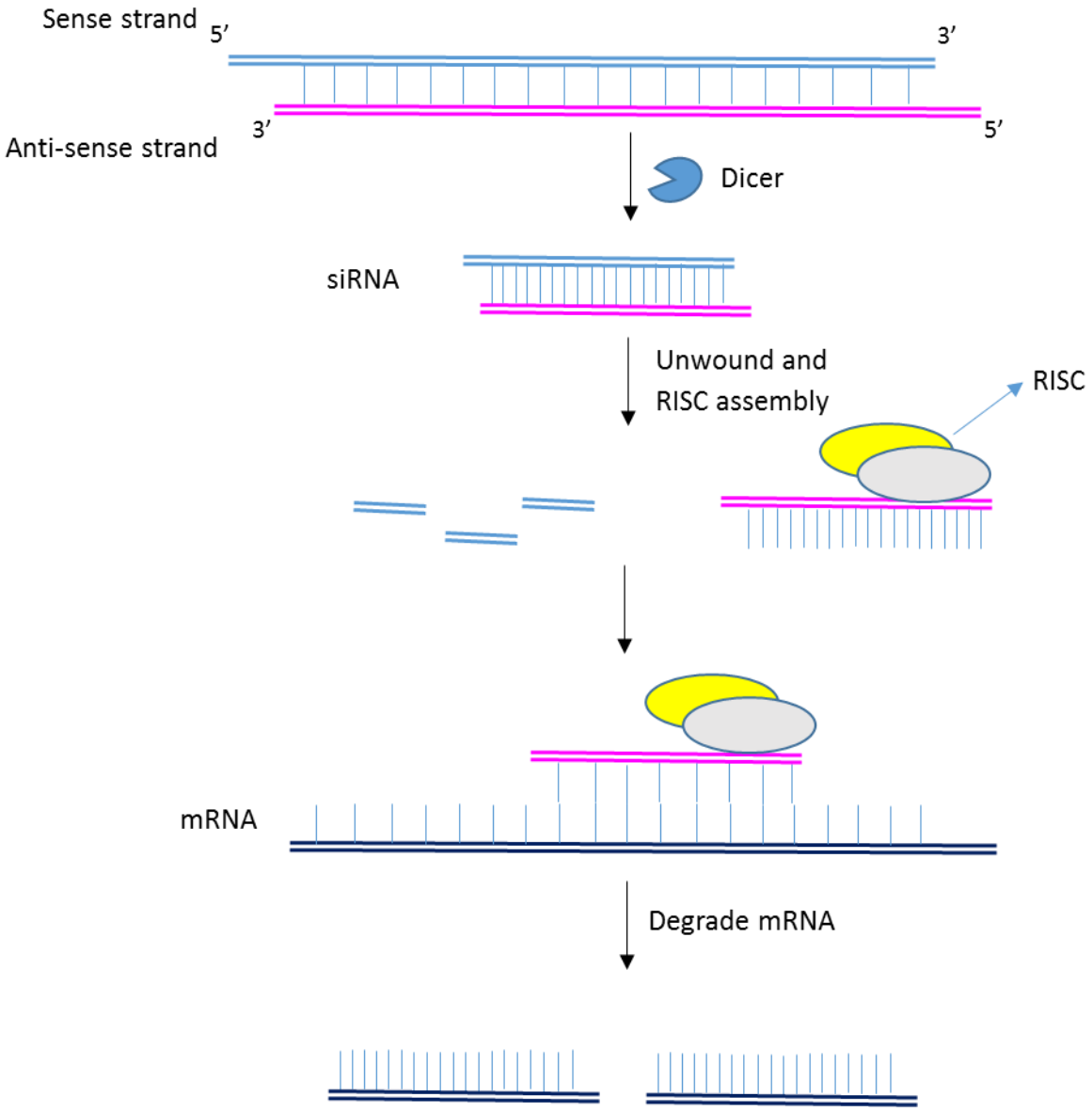


Figure 3.11: The siRNA pathway in intracellular

According to Carthew and Sontheimer on their article in 2009, in general, an enzyme called Dicer will initiate the siRNA pathway in which Dicer will cleave long double strand RNA into short double strand fragments of 20-30 nucleotides. Then siRNA is unwound into sense strand that will be degraded, and anti-sense strand that will incorporate with RISC (RNA-induced Silencing Complex). After the integration, siRNA base pairs will complementary with the target

mRNA to degrade and prevent it from the translation process.[56] In this research, to guarantee the intracellular release of siRNAs upon dicing, NPs were decorated with dicer substrate RNAs (DS RNAs).[57]

2. **Transfection into human breast cancer cell line (MDA-MB-231):**

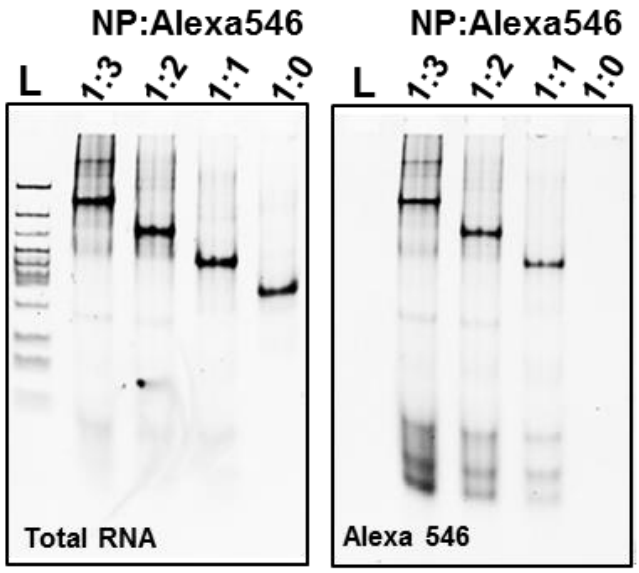


Figure 3.12: Native 7% PAGE experiment demonstrates sequential assembly of triangle with 1 µM, 2 µM, and 3 µM DNA-Alexa 546 oligodeoxynucleotide (ODN) per 1 µM RNA/DNA-center hybrid triangle NP. The gel was first scanned to detect signals from ODN-Alexa546 conjugates (right) then the gel was stained in EthBr for total nucleic acid imaging (L: DNA ladder). This gel also proofs that RNA can be produced with defined shape and stoichiometry as mentioned in chapter 1. We can see that when different concentrations of ODN-Alexa546 conjugate with the hybrid triangular nanoparticle, the shape of nanoparticle was not disrupted since each band was very precise at different concentrations of Alexa on the gel.

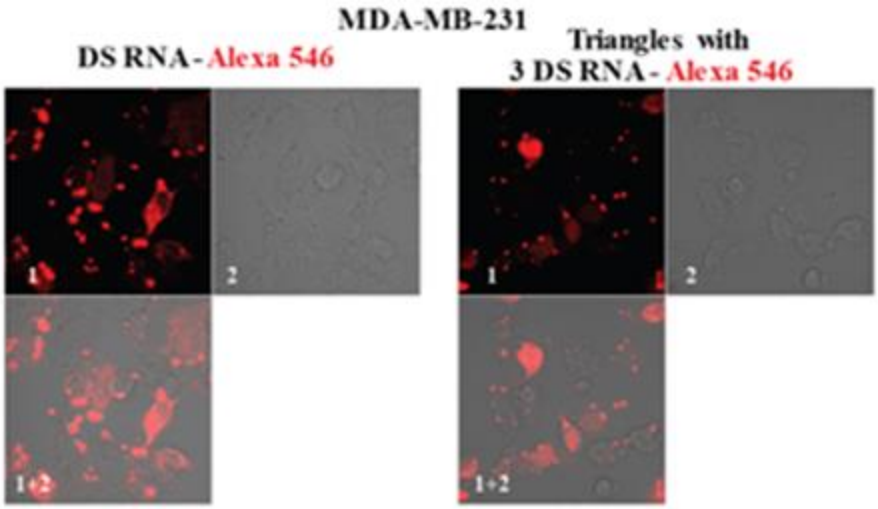


Figure 3.13: Fluorescent microscopy of non-fluorescent cells 24 hours post transfection with Alexa 546 labeled triangles (50 nM) (Performed by Dr. Kirill Afonin @ UNC CHARLOTTE).

To access the delivery of functionalized RNA/DNA-center hybrid triangular nanoparticles, all transfections were performed using human breast cancer cell line (MDA-MB-231). Figure 3.13 shows a fluorescent microscopy after 24 hours transfection the hybrid triangle carrying a specific DNA-sense-Alexa546 sequence and the equivalent concentrations of free DS RNA as a control to non-fluorescent cells. Here, the nuclei of cell was visualized with fluorophore dye and became blue. When the free DS RNA the DNA-sense-Alexa546 sequence was conjugated with the nanoparticle and transferred to the cell, the cells captured the red color of Alexa546. It indicates that Alexa546 can be visualized when conjugated with the nanoparticle in cell.

3. siRNA delivery into human breast cancer cell line (MDA-MB-231) expressing GFP:

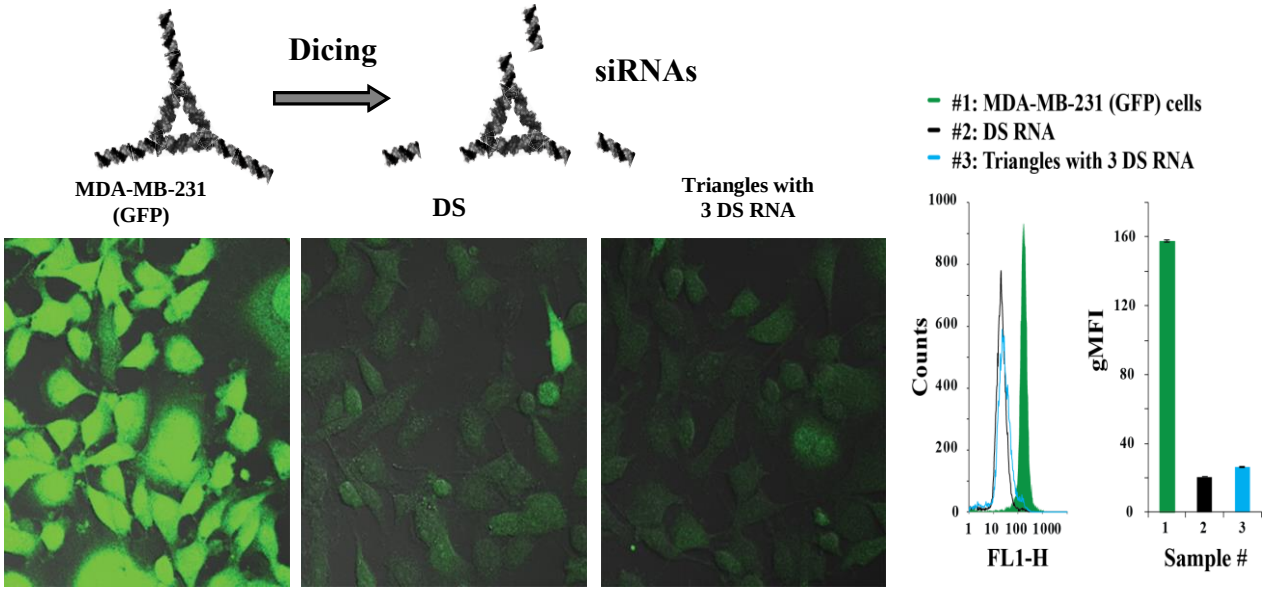


Figure 3.14: GFP-expressing cells were analyzed four days post-transfection with functionalized triangle (20 nM final) and DS RNA (60 nM final) by fluorescent microscope (left) and flow cytometry (right). Error bars denote +/- S.E.M (Performed by Dr. Kirill Afonin @ UNC CHARLOTTE).

Here, specific gene silencing experiments were carried out to confirm the successful dicer-assisted intracellular release of siRNA and further activation of RNA interference. Figure 3.14 shows a proof of GFP-expressing breast cancer cells (MDA-MB-231/GFP) were transfected with RNA/DNA-center hybrid triangular nanoparticles functionalized with three anti-GFP siRNAs and compared to free anti-GFP DS RNAs. The results show that GFP silencing of ~90% with comparable efficiencies for triangles and DS RNA at nanomolar concentrations. All the results clearly indicate that the RNA/DNA-center hybrid triangles can be used as scaffolds for controlled delivery of siRNAs and fluorescent probes to the diseased human cells.

VI. Construction and characterization of other polygons by using this artificial flexible 4x4x4 Us RNA Structural Module:

1. Polygons assembly evaluation on NATIVE PAGE gel:

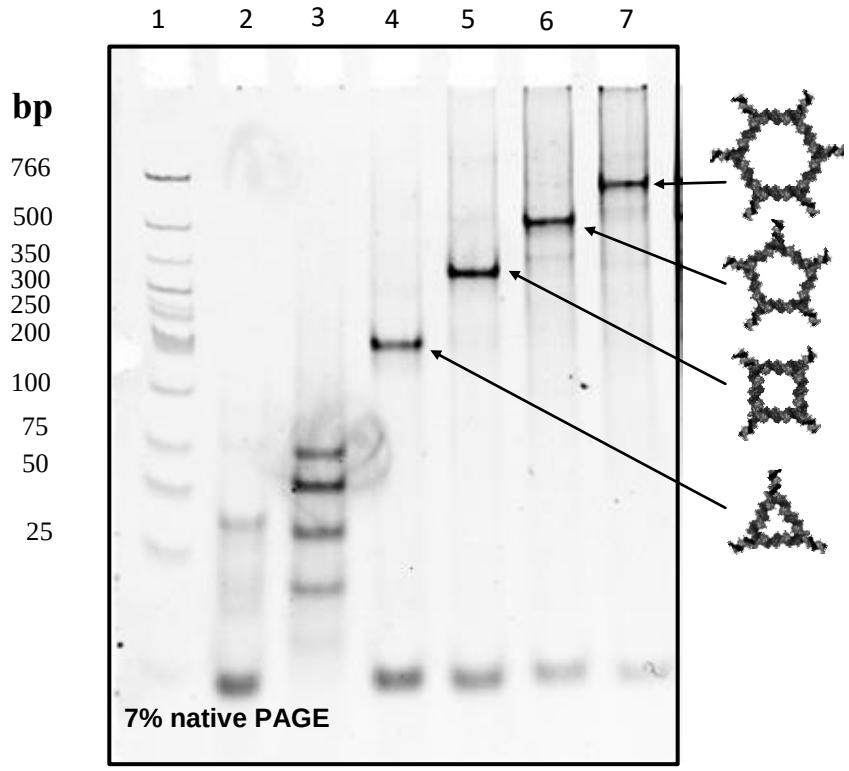


Figure 3.15: Self-assembly of RNA polygons evaluated by 7% native PAGE in 0.5x TBM running buffer.

This 7% native PAGE shows the assembling results of RNA polygons base on the migration of their molecular weight: Triangle, square, pentagon and hexagon. Well 2 is a mixture of six external strands (1 µM concentration of each): rT2, rT3, rT4, rS5, rP6, and rH7; since they are the shortest strands and have the same lengths, they migrate fastest in one band. Well 3 is a mixture of four internal strands (1 µM concentration of each): rT1, rS1, rP1, and rH1; each of them is different in length and therefore the gel shows four distinct bands: rT1 is the shortest strand and migrates furthest, then rS1, rP1, finally rH1 is the longest one and migrates slowest. Wells 4 to 7 are where RNA polygons are forming by adding each external strand to the mixture

of internal strands; as shown in section 1.4, each of the polygon is composed of different number of external strands and the propagation of internal strands. In well 4, rT1 was added to the mixture of external strands, then it corporates with rT2, rT3, and rT4 to form RNA triangle. Well 5 is the formation of RNA square where rS1, rT2, rT3, rT4 and rS5 are assembling together. When rP1 was added and assemble with rT2, rT3, rT4, rS5 and rP6, RNA pentagon was formed and shown a band on well 6. Finally, well 7 is where RNA hexagon was formed. This is the self-assembling of rH1 and all of the external strands in the mixture. Triangle nanoparticle migrates fastest with a lowest molecular weight about 200 bp; then, square has a molecular weight at about 300 bp, pentagon has a molecular weight at about 500 bp, and lastly, hexagon is the biggest about 760 bp and migrates slowest.

2. **RNA polygons assembly evaluation on AFM:** (RNA polygons were obtained in University of Nebraska Medical Center by Dr. Alexey Vrasnoslobotcev)

Again here, the evaluation of RNA polygons on native PAGE does not tell us that the nanoparticles we created are square, pentagon or hexagon shapes. It can be something that is a combination of RNA single strands. Getting the image on the AFM is one of the ways to check out the shape of these nanoparticles. Consistent with the theoretical model structures, the AFM images of RNA complexes revealed square, pentagon and hexagon shaped features as shown below:

i) RNA square:

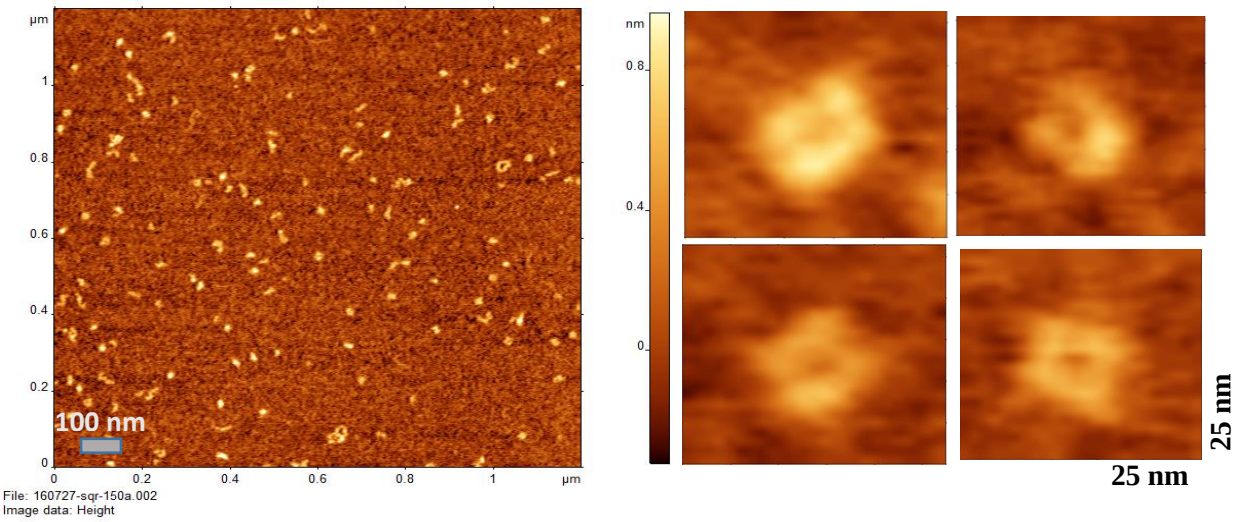


Figure 3.16: RNA square AFM images acquired in air at ambient temperature.

ii) RNA pentagon:

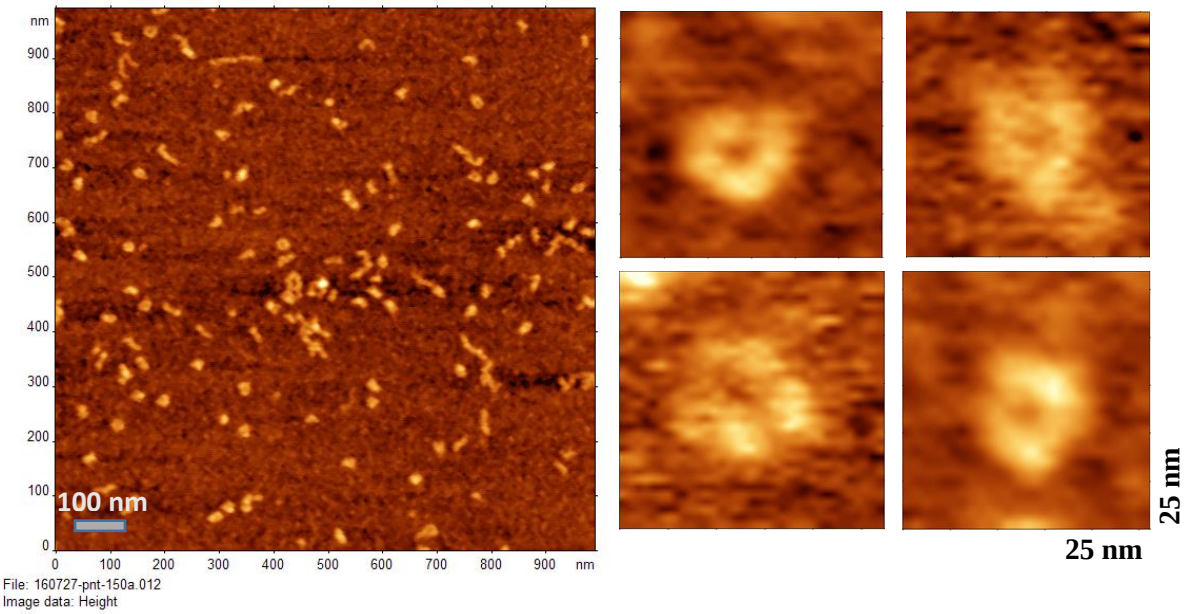


Figure 3.17: RNA pentagon AFM images acquired in air at ambient temperature.

iii) RNA hexagon:

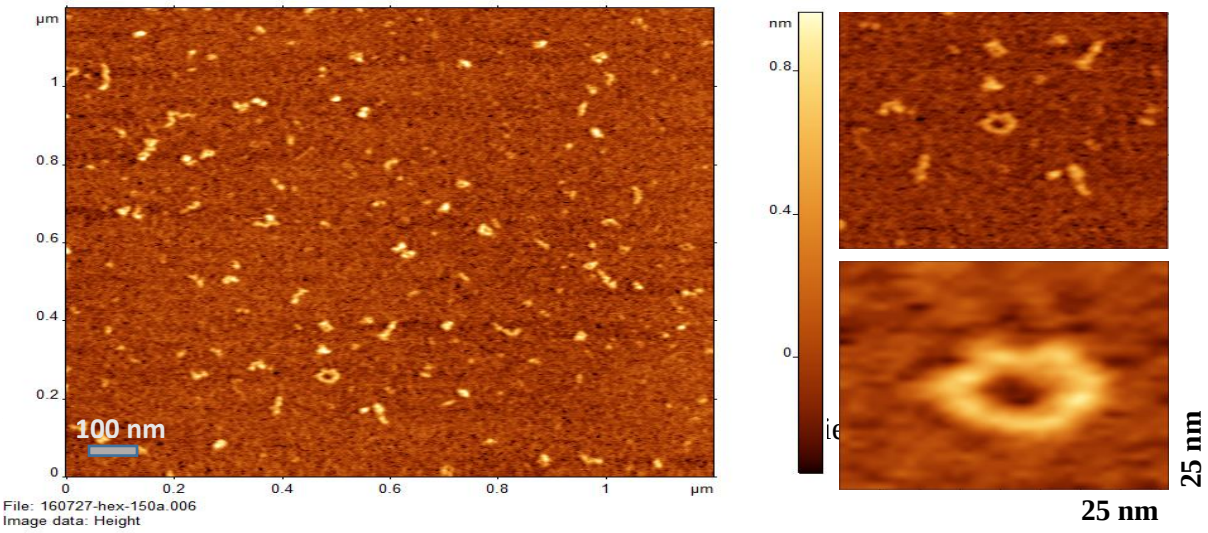


Figure 3.18: RNA hexagon AFM images acquired in air at ambient temperature.

3. Fine – tunable thermodynamic stabilities of RNA nanoparticles:

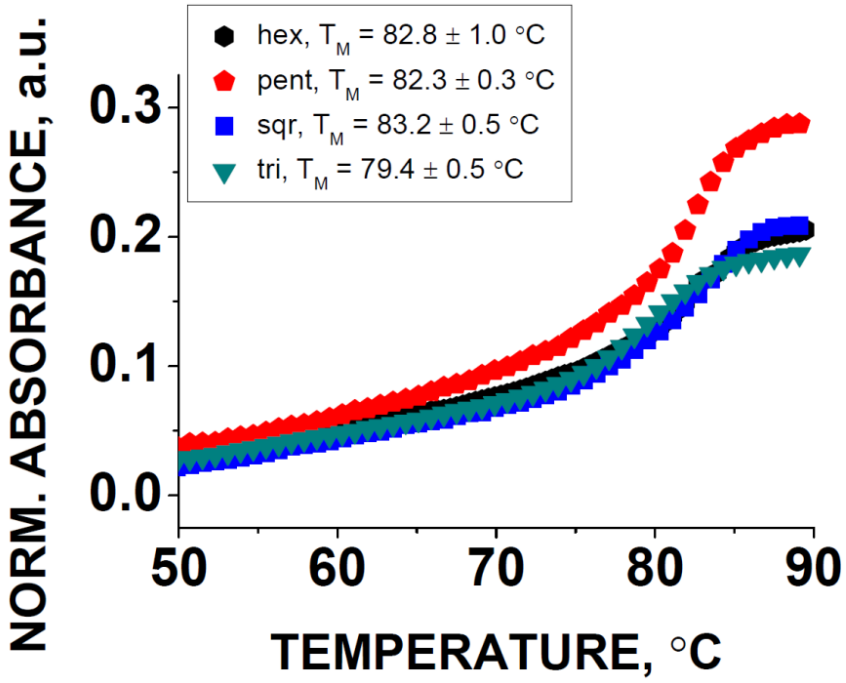


Figure 3.19: Thermal dynamic stability of RNA nanoparticles.

4. Fine-tunable chemical stabilities of nanoparticles:

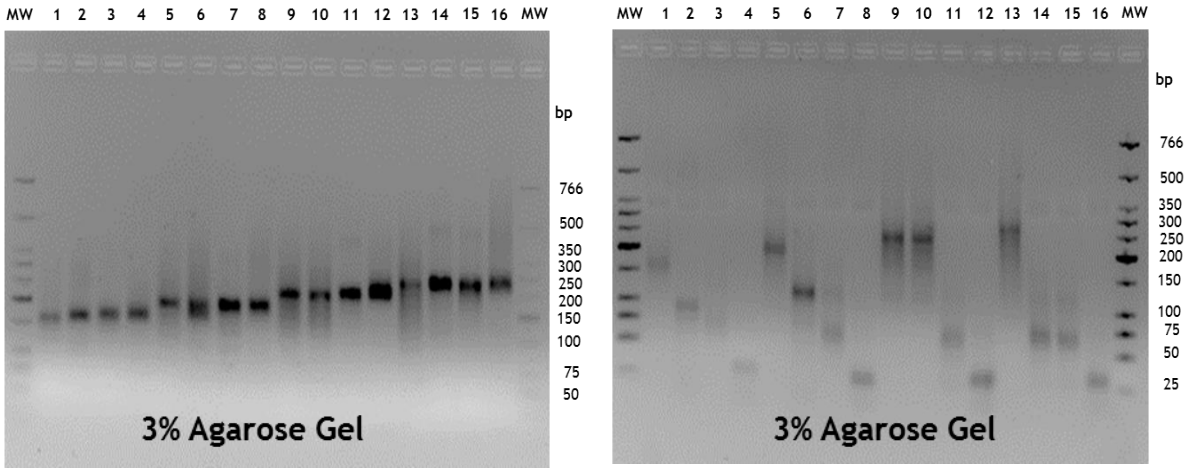


Figure 3.20: RNA/ DNA/ Hybrids nanostructures enzymatic stability assay. Nanoparticles were incubated at 37°C for 1 h in water as control (left) and in the presence of 20% FBS (right).

Wells:

Triangle:	1. DNA	2. DNA/RNA-center	3. RNA/DNA-center	4. RNA
Square:	1. DNA	2. DNA/RNA-center	3. RNA/DNA-center	4. RNA
Pentagon:	1. DNA	2. DNA/RNA-center	3. RNA/DNA-center	4. RNA
Hexagon:	1. DNA	2. DNA/RNA-center	3. RNA/DNA-center	4. RNA

This 3% agarose gel shows the enzymatic stability assay of RNA/ DNA/ Hybrids nanostructures. In this experiment, the nanoparticles were incubated for 1 hour in water as a control (left) and in the presence of 20% FBS (right). After visualizing the nucleic acid in EtBr solution, the band qualification from the gel was performed on ImageJ that will allow to determine the remaining fraction of corresponding triangles. The results suggest that the RNA nanoparticles is completely degraded while the RNA/DNA hybrids and DNA nanoparticle are found to be stable.

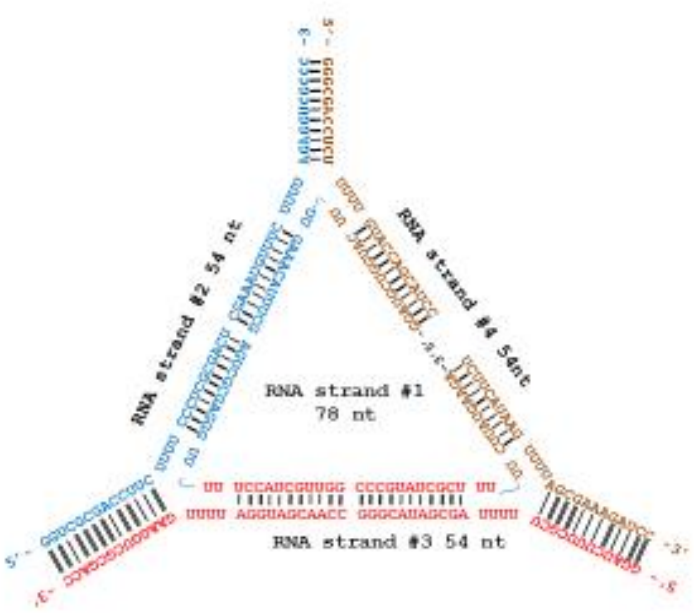
VII. Conclusion:

This project demonstrated the power of the rational design of triangle NPs based on flexible RNA 4x4x4 tetra-U helix linking module. Here, triangle NPs can be assembled from RNA, DNA or mixture of RNA/DNA thus allowing to change the physicochemical properties of the NPs. We found that the modulation of RNA and DNA strand composition makes it possible to engineer, in a *de novo* fashion, nanometer-scaled particles that are enzymatically resistant, thermodynamically stable, and potentially instrumental in the delivery of fluorescent probes and gene-silencing agents to cancer cells. Therefore, the triangular nano-scaffolds show great promise for biomedical applications due to their tunable immunostimulatory properties. The current project is under investigation with the ultimate goal of using the tetra U module for the construction other polygons made of RNA and DNA including squares, pentagons, and hexagons with application on nanomedicine.

Appendix:

A. <u>Design of RNA strands:</u>

i) RNA Triangular nanoparticle:



<u>Strand 1:</u>

RNA strand	5′- GGAUGCUGGUACUUUUGAAACAUUUCGAGUCGCGAGGGUUUUUCCAUCGUUGGC CCGUAUCGCUUUUUUCUUAUGAAGA – 3′
DNA strand	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTGAAACAT TTCGAGTCGC GAGGGTTTTT CCATCGTTGG CCCGTATCGC TTTTTCTTAT GAAGA - 3′
Tri_1 FRW prim	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTGAAACAT TTCGAGTCGC GAGG-3′
Tri_1 REV prim	5′- TCTTCATAAG AAAAAGCGAT ACGGGCCAAC GATGGAAAAA CCCTCGCGAC TCGAAATG - 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC - 3′
Tri_2 REV prim	5′- GGGCGACCTC TAAAAGAAAC ATTTCGAGTC GCGAGGGAAA AGAAGGTC – 3′

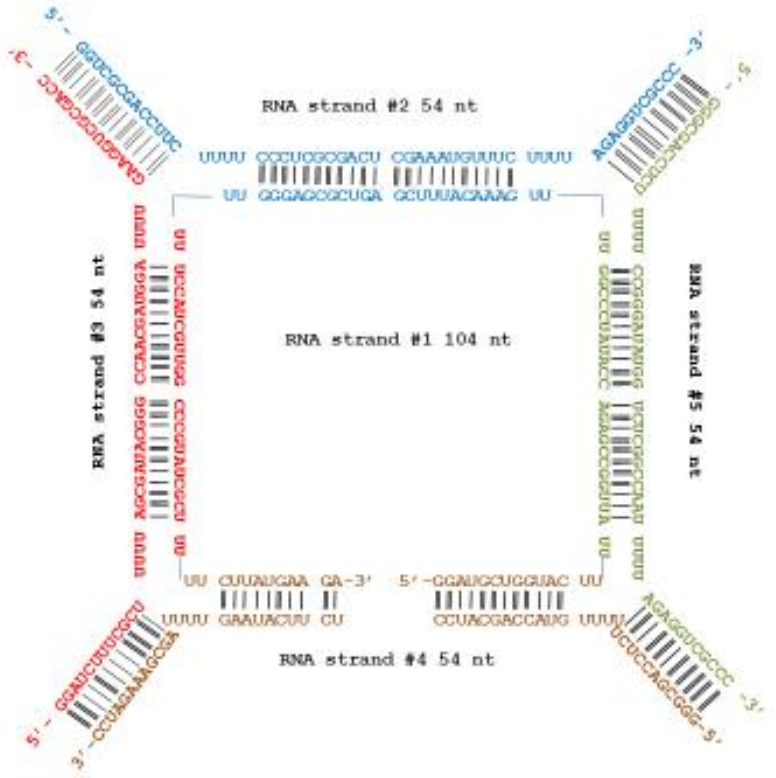
Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG – 3′
Tri_3 REV prim	5′- TCGCGACCTT CAAAATCCAT CGTTGGCCCG TATCGCTAAA AAGCGA -3′

Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- GATCTTTCGC TAAAACTTAT GAAGAGGATG CTGGTACAAA AAGAGGTC - 3′

ii) RNA Square nanoparticle:



Strand 1:

RNA strand	5′- GGAUGCUGGUACUUUUAUUGGCCGAGACCAUAUCCCGGUUUUGAAACAUUUCGAGU CGCGAGGGUUUUUCCAUCGUUGGCCCGUAUCGCUUUUUCUUAUGAAGA – 3′
DNA strand	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTG AAACATTTCG AGTCGCGAGG GTTTTTCCAT CGTTGGCCCG TATCGCTTTT TCTTATGAAG A- 3′
S_1 FRW prim	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTG AAACATTTCG AGTCGCGAGG – 3′
S_1 REV prim	5′- TCTTCATAAG AAAAAGCGAT ACGGGCCAAC GATGGAAAAA CCCTCGCGAC TCGAAATG – 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC - 3′
Tri_2 REV prim	5′- GGGCGACCTC TAAAAGAAAC ATTTCGAGTC GCGAGGGAAA AGAAGGTC – 3′

Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG – 3′
Tri_3 REV prim	5′- TCGCGACCTT CAAAATCCAT CGTTGGCCCG TATCGCTAAA AAGCGA -3′

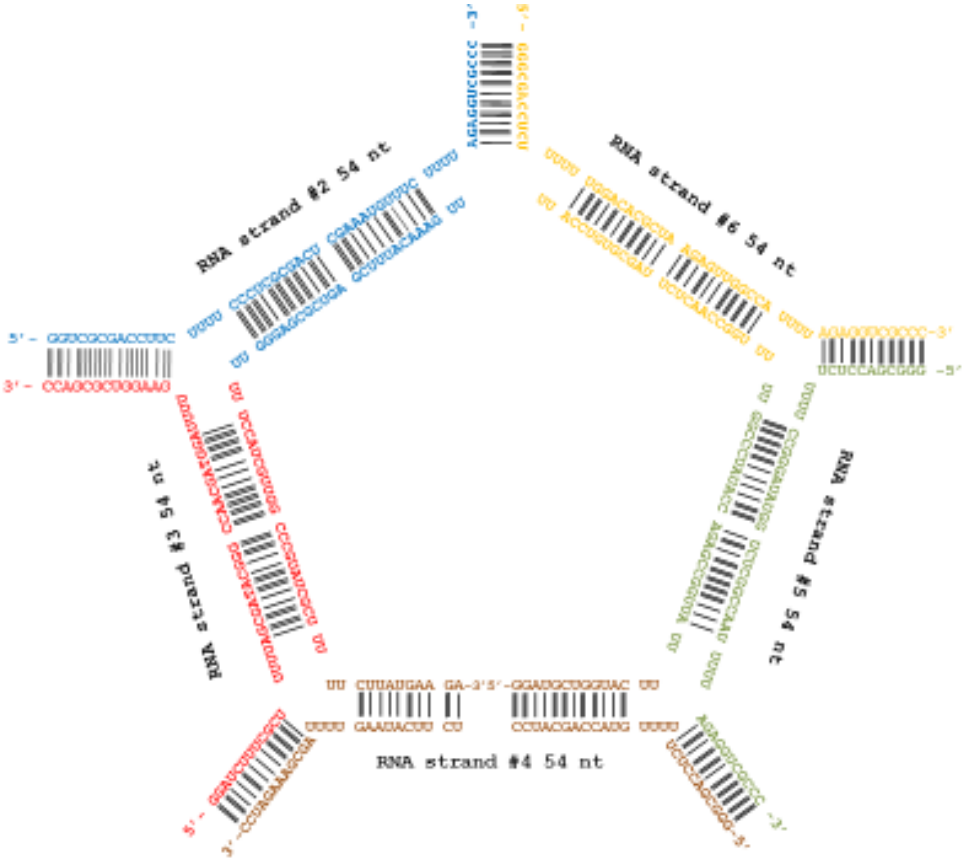
Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- GATCTTTCGC TAAAACTTAT GAAGAGGATG CTGGTACAAA AAGAGGTC - 3′

Strand 5:

RNA strand	5′- GGUCCGAAGCC UUUU CCGGGAUAUGG UCUCGGCCAAU UUUU UCCGAUGCAAU - 3′
DNA strand	5′ - TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCGGC CAATTTTTAG AGGTCGCCC - 3′
S_5 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCG - 3′
S_5 REV prim	5′- GGGCGACCTC TAAAAATTGG CCGAGACCAT ATCCCGG - 3′

iii) RNA pentagon nanoparticle:



Strand 1:

RNA strand	5′- **GGAUGCUGGUACUUUUAUUGGCCGAGACCAUAUCCCGG UUUU UGGCCAACUCUUAGCGUGUCCAUUUUGAAACAUUUCGAGUCGCGAGGGUUUUUCCA UCGUUGGCCCGUAUCGCUUUUUCUUAUGAAGA – 3′**
DNA strand	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTT GGCCAACTCT TAGCGTGTCC ATTTTGAAAC ATTTCGAGTC GCGAGGGTTT TTCCATCGTT GGCCCGTATC GCTTTTTCTT ATGAAGA – 3′
P_1 FRW prim	5′- TAATACGACTCACTATAGGATGCTGGTACTTTTATTGGCCGAGACCATATCC – 3′
Inner 1	ACTCTTAGCGTGTCCATTTTGAAACATTTCGAGTCGCGAGGGTTTTTCCATC
Inner 2	ATAACCGGCTCTGGTATAGGGCCAAAAACCGGTTGAGAATCGCACAGGTAAAAC
P_1 REV prim	5′- AGCGCTCCCAAAAAGGTAGCAACCGGGCATAGCGAAAAAGAATACTTCT – 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC – 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC – 3′
Tri_2 REV prim	5′- GGGCGACCTC TAAAAGAAAC ATTTCGAGTC GCGAGGGAAA AGAAGGTC – 3′

Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA – 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG – 3′
Tri_3 REV prim	5′- TCGCGACCTT CAAAATCCAT CGTTGGCCCG TATCGCTAAA AAGCGA -3′

Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- GATCTTTCGC TAAAACTTAT GAAGAGGATG CTGGTACAAA AAGAGGTC - 3′

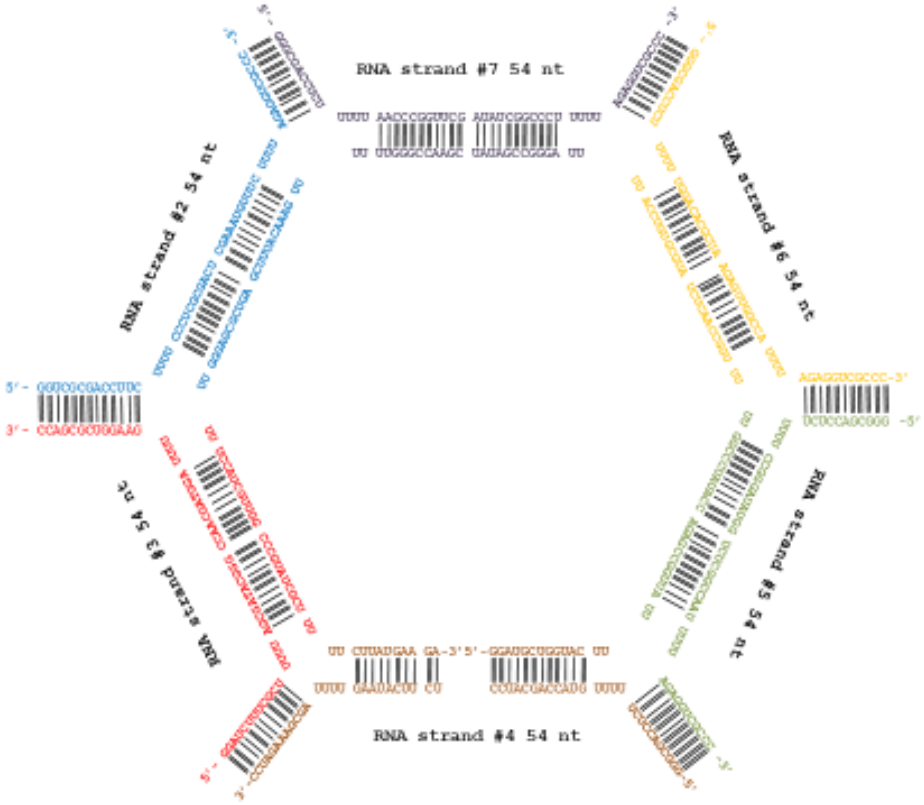
Strand 5:

RNA strand	**5′- GGUCCGAAGCC UUUU CCGGGAUAUGG UCUCGGCCAAU UUUU UCCGAUGCAAU - 3′**
DNA strand	**5′ -** TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCGGC CAATTTTTAG AGGTCGCCC – 3′
S_5 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCG – 3′
S_5 REV prim	5′- GGGCGACCTC TAAAAATTGG CCGAGACCAT ATCCCGG - 3′

Strand 6:

RNA strand	**5′- GGGCGACCUCU UUUU UGGACACGCUA AGAGUUGGCCA UUUU AGAGGUCGCCC - 3′**
DNA strand	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG GCCATTTTAG AGGTCGCC - 3′
P_6 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG – 3′
P_6 REV prim	5′- GGCGACCTCT AAAATGGCCA ACTCTTAGCG TGTCC - 3′

iv) RNA Hexagon nanoparticle



Strand 1:

RNA strand	**5′–** **TAATACGACTCACTATA**GGAUGCUGGUACUUUUAUUGGCCGAGACCAUAUCCCGGU **U UU UGGCCAACUCU UAGCGUGUCCA UU** UUAGGGCCGAUAUCGAACCGGGUUUUUUGAAACAUUUCGAGUCGCGAGGGUUUUUC CAUCGUUGGCCCGUAUCGCUUUUUCUUAUGAAGA **– 3′**
DNA strand	5′– TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTT GGCCAACTCT TAGCGTGTCC ATTTTAGGGC CGATATCGAA CCGGGTTTTT TGAAACATTT CGAGTCGCGA GGGTTTTTCC ATCGTTGGCC CGTATCGCTT TTTCTTATGA AGA – 3′
H_1 FRW prim	TAATACGACTCACTATAGGATGCTGGTACTTTTATTGGCCGAGACCATATCCCGGT TT – 3′
Inner 1	CCATTTTAGGGCCGATATCGAACCGGGTTTTTTGAAACATTTCGAGTCGCGAGGGT TT
Inner 2	GCTCTGGTATAGGGCCAAAAACCGGTTGAGAATCGCACAGGTAAAATCCCGGCTAT AGC
H_1 REV prim	5′– AAAGCTCAGCGCTCCCAAAAAGGTAGCAACCGGGCATAGCGAAAAAGAATACTTCT – 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC - 3′
Tri_2 REV prim	5′- GGGCGACCTC TAAAAGAAAC ATTTCGAGTC GCGAGGGAAA AGAAGGTC – 3′

Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG – 3′
Tri_3 REV prim	5′- TCGCGACCTT CAAAATCCAT CGTTGGCCCG TATCGCTAAA AAGCGA -3′

Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- GATCTTTCGC TAAAACTTAT GAAGAGGATG CTGGTACAAA AAGAGGTC - 3′

Strand 5:

RNA strand	**5′- GGUCCGAAGCC UUUU CCGGGAUAUGG UCUCGGCCAAU UUUU UCCGAUGCAAU - 3′**
DNA strand	**5′ -** TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCGGC CAATTTTTAG AGGTCGCCC – 3′
S_5 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCG – 3′
S_5 REV prim	5′- GGGCGACCTC TAAAAATTGG CCGAGACCAT ATCCCGG - 3′

Strand 6:

RNA strand	**5′- GGGCGACCUCU UUUU UGGACACGCUA AGAGUUGGCCA UUUU AGAGGUCGCCC - 3′**
DNA strand	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG GCCATTTTAG AGGTCGCC - 3′
P_6 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG – 3′
P_6 REV prim	5′- GGCGACCTCT AAAATGGCCA ACTCTTAGCG TGTCC - 3′

Strand 7:

RNA strand	5′- **GGGCGACCUCU UUUU AACCCGGUUCG AUAUCGGCCCU UUUU AGAGGUCGCCC -3′**
DNA strand	**5′-** TAATACGACT CACTATAGGGCGACCTCT TTTT AACCCGGTTCG ATATCGGCCCT TTTT AGAGGTCGCCC – 3′
H_7 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTAACCCGGT TCGATATCGG – 3′
H_7 REV prim	5′- GGGCGACCTC TAAAAAGGGC CGATATCGAA CCGGGTT - 3′

B. Functionalized RNA with siRNA:

i) RNA triangle:

Strand 1:

RNA strand	**5′-** **GGAUGCUGGUACUUUUGAAACAUUUCGAGUCGCGAGGGUUUUUCCAUCGUUGGC CCGUAUCGCUUUUUCUUAUGAAGA – 3′**
DNA strand	**5′-** TAATACGACT CACTATAGGA TGCTGGTACT TTTGAAACAT TTCGAGTCGC GAGGGTTTTT CCATCGTTGG CCCGTATCGC TTTTTCTTAT GAAGA – 3′
Tri_1 FRW prim	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTGAAACAT TTCGAGTCGC GAGG-3′
Tri_1 REV prim	5′- TCTTCATAAG AAAAAGCGAT ACGGGCCAAC GATGGAAAAA CCCTCGCGAC TCGAAATG – 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU – 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC **CGGTGGTGCA GATGAACTTC AGGGT** – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC – 3′
Tri_2 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA GAAACATTTC GAGTCGCGAG GGAAAAGAAG GTC– 3′

Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA CGGUGGUGCA GAUGAACUUC AGGGU – 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG –3′
Tri_3 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGTCGCG ACCTTCAAAA TCCATCGTTG GCCCGTATCG CTAAAAAGCG A –3′

Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGATCT TTCGCTAAAA CTTATGAAGA GGATGCTGGT ACAAAAAGAG GTC- 3′

ii) RNA Square:

Strand 1:

RNA strand	5′- **GGAUGCUGGUACUU**UUAUUGGCCGAGACCAUAUCCCGGUU**UUGAAACAUUUCGAGU CGCGAGGGUU**UUUCCAUCGUUGGCCCGUAUCGCUUU**UUCUUAUGAAGA – 3′**
DNA strand	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTG AAACATTTCG AGTCGCGAGG GTTTTTCCAT CGTTGGCCCG TATCGCTTTT TCTTATGAAG A- 3′
S_1 FRW prim	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTG AAACATTTCG AGTCGCGAGG′- 3′
S_1 REV prim	5′- TCTTCATAAG AAAAAGCGAT ACGGGCCAAC GATGGAAAAA CCCTCGCGAC TCGAAATG- 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC **CGGTGGTGCA GATGAACTTC AGGGT** – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC - 3′
Tri_2 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA GAAACATTTC GAGTCGCGAG GGAAAAGAAG GTC- 3′

Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG –3′
Tri_3 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGTCGCG ACCTTCAAAA TCCATCGTTG GCCCGTATCG CTAAAAAGCG A –3′

Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGATCT TTCGCTAAAA CTTATGAAGA GGATGCTGGT ACAAAAAGAG GTC- 3′

Strand 5:

RNA strand	5′- **GGGCGACCUCU UUUU CCGGGAUAUGG UCUCGGCCAAU UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU** –3′
DNA strand	**5′ -** TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCGGC CAATTTTTAG AGGTCGCCC **CGGTGGTGCA GATGAACTTC AGGGT** – 3′
S_5 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCG – 3′
S_5 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA ATTGGCCGAG ACCATATCCC GG - 3′

iii) RNA pentagon:

Strand 1:

RNA strand	5′- **GGAUGCUGGUACUU**UUAUUGGCCGAGACCAUAUCCCGG UUUU UGGCCAACUCU UAGCGUGUCCA UU**UUGAAACAUUUCGAGUCGCGAGGGUU**UUUCCAUCGUUGGCCCGUAUCGCUUUUU **CUUAUGAAGA – 3′**
DNA strand	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTT GGCCAACTCT TAGCGTGTCC ATTTTGAAAC ATTTCGAGTC GCGAGGGTTT TTCCATCGTT GGCCCGTATC GCTTTTTCTT ATGAAGA – 3′
P_1 FRW prim	5′- TAATACGACTCACTATAGGATGCTGGTACTTTTATTGGCCGAGACCATATCC – 3′
Inner 1	ACTCTTAGCGTGTCCATTTTGAAACATTTCGAGTCGCGAGGGTTTTTCCATC
Inner 2	ATAACCGGCTCTGGTATAGGGCCAAAAACCGGTTGAGAATCGCACAGGTAAAAC
P_1 REV prim	5′- AGCGCTCCCAAAAAGGTAGCAACCGGGCATAGCGAAAAAGAATACTTCT – 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC **CGGTGGTGCA GATGAACTTC AGGGT** – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC - 3′
Tri_2 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA GAAACATTTC GAGTCGCGAG GGAAAAGAAG GTC- 3′

Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG –3′
Tri_3 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGTCGCG ACCTTCAAAA TCCATCGTTG GCCCGTATCG CTAAAAAGCG A –3′

Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGATCT TTCGCTAAAA CTTATGAAGA GGATGCTGGT ACAAAAAGAG GTC- 3′

Strand 5:

RNA strand	5′- **GGGCGACCUCU UUUU CCGGGAUAUGG UCUCGGCCAAU UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU** –3′
DNA strand	**5′ -** TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCGGC CAATTTTTAG AGGTCGCCC **CGGTGGTGCA GATGAACTTC AGGGT** – 3′
S_5 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCG – 3′
S_5 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA ATTGGCCGAG ACCATATCCC GG - 3′

Strand 6:

RNA strand	5′- GGGCGACCUCU UUUU UGGACACGCUA AGAGUUGGCCA UUUU AGAGGUCGCCC **CGGUGGUGCA GAUGAACUUC AGGGU** - 3′
DNA strand	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG GCCATTTTAG AGGTCGCC CGGTGGTGCA GATGAACTTC AGGGT - 3′
P_6 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG - 3′
P_6 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGCGA CCTCTAAAAT GGCCAACTCT TAGCGTGTCC - 3′

iv) RNA Hexagon:

Strand 1:

RNA strand	5′- TAATACGACTCACTATA**GGAUGCUGGUACUU**UUAUUGGCCGAGACCAUAUCCCGGU U UU UGGCCAACUCU UAGCGUGUCCA UU UU AGGGCCGAUAU CGAACCGGGUU UU**UUGAAACAUUUCGAGUCGCGAGGGUU**UUUCCAUCGUUGGCCCGUAUCGCUUUUU CUUAUGAAGA - 3′
DNA strand	5′- TAATACGACT CACTATAGGA TGCTGGTACT TTTATTGGCC GAGACCATAT CCCGGTTTTT GGCCAACTCT TAGCGTGTCC ATTTTAGGGC CGATATCGAA CCGGGTTTTT TGAAACATTT CGAGTCGCGA GGGTTTTTCC ATCGTTGGCC CGTATCGCTT TTTCTTATGA AGA - 3′
P_1 FRW prim	5′-TAATACGACTCACTATAGGATGCTGGTACTTTTATTGGCCGAGACCATATCCCGGTTT - 3′
Inner 1	CCATTTTAGGGCCGATATCGAACCGGGTTTTTTGAAACATTTCGAGTCGCGAGGGTTT
Inner 2	GCTCTGGTATAGGGCCAAAAACCGGTTGAGAATCGCACAGGTAAAATCCCGGCTATAGC
P_1 REV prim	5′- AAAGCTCAGCGCTCCCAAAAAGGTAGCAACCGGGCATAGCGAAAAAGAATACTTCT - 3′

Strand 2:

RNA strand	**5′- GGUCGCGACC UUCUUUU CCCUCGCGACU CGAAAUGUUUC UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGCGACTCG AAATGTTTCT TTTAGAGGTC GCCC **CGGTGGTGCA GATGAACTTC AGGGT** – 3′
Tri_2 FRW prim	5′- TAATACGACTCACTATA GGTCGCGACC TTCTTTTCCC TCGC - 3′
Tri_2 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA GAAACATTTC GAGTCGCGAG GGAAAAGAAG GTC- 3′

Strand 3:

RNA strand	**5′- GGAUCUUUCGCU UUUU AGCGAUACGGG CCAACGAUGGA UUUU GAAGGUCGCGA CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGGCCA ACGATGGATT TTGAAGGTCG CGA CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_3 FRW prim	5′- TAATACGACTCACTATA GGATCTTTCG CTTTTTAGCG ATACGGG –3′
Tri_3 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGTCGCG ACCTTCAAAA TCCATCGTTG GCCCGTATCG CTAAAAAGCG A -3′

Strand 4:

RNA strand	**5′- GGGCGACCUCU UUUU GUACCAGCAUC CUCUUCAUAAG UUUU AGCGAAAGAUC CGGUGGUGCA GAUGAACUUC AGGGU - 3′**
DNA strand	**5′-** TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AGCATCCTCT TCATAAGTTT TAGCGAAAGA TC CGGTGGTGCA GATGAACTTC AGGGT – 3′
Tri_4 FRW prim	5′- TAATACGACTCACTATA GGGCGACCTC TTTTTGTACC AG – 3′
Tri_4 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGATCT TTCGCTAAAA CTTATGAAGA GGATGCTGGT ACAAAAAGAG GTC- 3′

Strand 5:

RNA strand	5′- **GGGCGACCUCU UUUU CCGGGAUAUGG UCUCGGCCAAU UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU** -3′
DNA strand	**5′ -** TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCGGC CAATTTTTAG AGGTCGCCC **CGGTGGTGCA GATGAACTTC AGGGT** – 3′
S_5 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTCCGGGATA TGGTCTCG – 3′
S_5 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA ATTGGCCGAG ACCATATCCC GG - 3′

Strand 6:

RNA strand	5′- **GGGCGACCUCU UUUU UGGACACGCUA AGAGUUGGCCA UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU** - 3′
DNA strand	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG GCCATTTTAG AGGTCGCC CGGTGGTGCA GATGAACTTC AGGGT - 3′
P_6 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTTGGACACG CTAAGAGTTG – 3′
P_6 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGCGA CCTCTAAAAT GGCCAACTCT TAGCGTGTCC - 3′

Strand 7:

RNA strand	5′- **GGGCGACCUCU UUUU AACCCGGUUCG AUAUCGGCCCU UUUU AGAGGUCGCCC CGGUGGUGCA GAUGAACUUC AGGGU** -3′
DNA strand	**5′-** GGGCGACCTC TTTTTAACCC GGTTCGATAT CGGCCCTTTT TAGAGGTCGC CCCGGTGGTG CAGATGAACT TCAGGGT- 3′
H_7 FRW prim	5′- TAATACGACT CACTATAGGG CGACCTCTTT TTAACCCGGT TCGATATCGG- 3′
H_7 REV prim	5′- ACCCTGAAGT TCATCTGCAC CACCGGGGCG ACCTCTAAAA AGGGCCGATA TCGAACCGGG TT - 3′

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